ALPHA-LINOLENIC ACID CONFERS NEUROPROTECTION AND IMPROVES BEHAVIORAL DEFICITS AFTER SOMAN EXPOSURE: INVOLVEMENT OF NEUROGENESIS THROUGH AN mTOR-MEDIATED PATHWAY

by

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ABSTRACT

Title of Dissertation: Alpha-linolenic acid confers neuroprotection and improves behavioral deficits after soman exposure: involvement of neurogenesis through an mTOR-mediated pathway

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Soman, an organophosphorous (OP) compound, is a chemical warfare agent that irreversibly inhibits acetylcholinesterase in the periphery and central nervous system. Soman induces *status epilepticus* leading to brain damage associated with long-term cognitive and behavioral deficits. Current countermeasures are only effective when administered shortly after exposure to nerve agents and there are no therapies that protect against the long-term cognitive and behavioral impairments, which call for the search of new and efficacious therapies.

Alpha linolenic acid (LIN) is an essential omega-3 polyunsaturated fatty acid (PUFA) found in green leaves, seed oil (flaxseeds), beans and walnuts. The compound can be purchased over-the-counter and has no side effects. Administration of a single dose of alpha-linolenic acid has been demonstrated to exert a potent neuroprotective effect against neuronal degeneration induced by kainic acid, transient global ischemia and

organophosphates *in vivo*. However, the molecular mechanism(s) of LIN-induced neuroprotection administered after soman have not been delineated.

In the present study, we investigated the neuroprotective and neurorestorative effects of LIN treatment in young adult Sprague-Dawley male rats. Rats were pretreated with HI-6 (125 mg /kg, i.p.), a compound that reactivates acetylcholinesterase, followed by an injection with soman (180 ug/kg s.c.; 1.6xLD_{50}), and additional life-saving treatment with atropine methyl nitrate (2 mg/kg, i.m.), a muscarinic cholinergic antagonist that blocks the peripheral effects of the induced cholinergic crisis, and diazepam (10mg/kg, sc), a benzodiazepine that attenuates seizures in soman-treated animals. LIN (500 nmol/kg i.v) was administered 30 min, 3 days and 7 days prior to (pretreatment) or 30 min, 3 days and 7 days after (post-treatment) soman exposure. Our findings demonstrate that the neuropathological, pathophysiological and behavioral effects of soman exposure can be attenuated by treatment with LIN only *after* soman exposure, suggesting that the timing of LIN administration is critical to exert the neuroprotective and neurorestorative effects in the brain.

The present study was also aimed to determine possible molecular mechanisms of alpha-linolenic acid-induced neuroprotection in the hippocampus in parallel with a hippocampal-dependent learning and memory paradigm after soman exposure. We show that soman exposure increases the endogenous expression levels of mature brain-derived neurotrophic factor (BDNF) in the hippocampus but BDNF was mostly localized in non-neuronal cells in the dentate gyrus (DG) of the hippocampus as shown by immunohistochemistry; the increase in BDNF had no effect on downstream signaling cascades. In contrast, administration of alpha-linolenic acid injected intravenously at 30

min, 3 days and 7 days after soman or saline exposure increases the endogenous expression levels of mature BDNF mainly in neurons in the DG of the hippocampus. Western blot analysis of Akt and mammalian target of rapamycin (mTOR) demonstrated a significant activation of Akt and mTOR only in LIN-treated groups of animals, resulting in enhanced neurogenesis in the subgranular zone of the DG and increased retention latency in the passive avoidance task. Administration of rapamycin, an inhibitor of mTORC1, blocked the observed LIN-induced neurogenesis and the LIN-induced improvement on the passive avoidance task. These data support a model in which LIN increases the endogenous expression protein levels of BDNF which in turn activates its cognate receptor, TrkB and downstream signaling of Akt and mTOR to enhance hippocampal neurogenesis and improve cognitive performance. These results suggest that LIN could be used as a therapeutic agent to restore cognitive and behavioral function after exposure to nerve agents.

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LIST OF ABREVIATION

ACh, acetylcholine

AChE, acetylcholinesterase

Akt/PKB, protein kinase B

AMPAs, alkyl methylphosphonic acids

BDNF, brain-derived neurotrophic factor

BrdU, 5-Bromo–deoxyuridine

CA1, region CA1 of hippocampus, *cornus ammonis 1*

CA3, region CA3 of hippocampus, *cornus ammonis 3*

cAMP , cyclic AMP

CaRF , Ca^{2+}-response factor

CaMKII, Ca^{2+} /calmodulin kinase II

CNS, central nervous system

CREB, cAMP response element (CRE)-binding protein

CS, conditioned stimulus

DCX, Doublecortin

DG, dentate gyrus of hippocampus

DGC, dentate granule cells

DHA, docosahexaenoic acid

EPA, ecoisapentaenoic acid

ERK, extracellular signal-regulated kinases

GCL, Granule cell layer

LIN, alpha-linolenic acid

mAChR, muscarinic acetylcholine receptor

MAPK, mitogen-activated protein kinase

MPA, methyl phosphonic acid

mTOR, mammalian target of rapamycin

mTORC1, mTOR complex 1

mTORC2, mTOR complex 2

NMDA, N-methyl-D-aspartate

OP, organophosphorous compounds

PLC, phospholipase C

PNS, peripheral nervous system

PTSD, post-traumatic stress disorder

PUFA, polyunsaturated fatty acid

SVZ, subventricular zone

TrkB, Tropomyosin related kinase B receptor

USF, upstream stimulatory factor 1/2

US, unconditioned stimulus

Chapter 1: General Introduction

Organophosphorous compounds (OP) such as soman (O-1,2,2-trimethylpropylmethyl-phosphono-fluoridate) are nerve agents used in chemical warfare and are weapons of mass destruction. These compounds are extremely hazardous due to their high toxicity and superior penetration into the human body via the skin, inhalation, and through the bloodstream. The underlying mechanism of OP-induced toxicity is the irreversible inhibition of acetylcholinesterase (AChE), which in turns leads to a plethora of signs and symptoms including *status epilepticus* and death. The neuropathology leads to severe cognitive performance, including long-term cognitive and behavioral deficits in learning and memory and depression in human and animal survivors.

In this study, we employ soman as a model of OP-induced neurotoxicity to examine the neuroprotective efficacy of alpha-linolenic acid (LIN), an essential polyunsaturated fatty acid (PUFA). LIN is found in vegetable oils, green leaves, seed oil (flaxseeds), beans, walnuts and can be purchased over-the-counter and has a wide safety margin. Recent findings from our lab demonstrated that a single intravenous administration of LIN exerts a potent protective effect against soman-induced neuropathology, although the mechanism(s) of protection are not yet clear. Our lab has also previously shown that three injections of LIN, but not a single injection, increase brain-derived neurotrophic factor (BDNF) levels in the cortex and hippocampus and is associated with increased synaptogenesis, neurogenesis, synaptic function and exert an anti-depressant effect in naïve rats. Activity-dependent release of BDNF could activate TrkB receptors in a paracrine or autocrine loop, which in turn would lead to the activation of downstream signaling pathways such as Akt, ERK and the mammalian target of

rapamycin (mTOR), a serine/threonine kinase involved in a broad array of processes in the brain. Therefore, understanding the molecular and cellular mechanisms activated by alpha-linolenic acid will provide new insights into how LIN restores function after brain injury.

The purpose of this dissertation is to investigate the role of three doses of LIN administered after or before soman exposure on functional outcome and to determine the long-term neuroprotective effect and the cell signaling pathways that may be mediated by LIN against soman-induced neurodegeneration and cognitive deficits (decline in learning and memory, and depression) in adult male Sprague-Dawley rats. My central hypothesis is that the increase in mature BDNF levels in the hippocampus by LIN activates its cognate receptor, TrkB, leading to the activation of Akt and the mTOR complex 1 (mTORC1) which in turn increases neurogenesis to restore cognitive performance in soman-induced neuropathology. There are three aims to address this hypothesis:

Aim 1. To determine histologic and behavioral measures in alpha-linolenic acid-treated rats following soman injection. Soman induces neuronal cell death in several well-established brain regions. Previous results from our group showed convincing evidence that a single dose of alpha-linolenic acid is efficacious in protecting against soman-induced neuropathology. We also showed that three sequential doses of LIN increases the endogenous expression of mature BDNF in the cortex and hippocampus and exerts an antidepressant-like effect in naïve mice. Herein, I want to investigate whether the soman-induced neuronal degeneration is associated with long-term cognitive deficits (learning and memory, and depression) in our model of soman-induced neurotoxicity and propose to investigate whether administration of three doses of LIN intravenously given

prior or after soman exposure increases neuronal survival and improves functional outcome.

Aim 2. To determine neurogenesis induced by alpha-linolenic acid after soman exposure. Cognitive deficits have been reported in OP-treated rats and impairment in spatial memory after soman exposure has been associated with reduced neurogenesis. Increased neurogenesis triggers several mechanisms that may promote restoration and enhancement of hippocampal functions after soman exposure. Since the administration of three doses of LIN has been previously shown to increase neurogenesis in the subgranular zone of the dentate gyrus in naïve mice, I propose to investigate the effect of soman on neurogenesis in the hippocampus. I also want to determine whether the protective effect of alpha-linolenic acid is associated with an enhancement in neurogenesis after soman exposure.

Aim 3. To determine a possible intracellular signaling pathway mediated by LIN against soman-induced neuropathology. Soman exposure results in profound cognitive deficits in humans and rodents. The mTOR signaling pathway is known to mediate several cellular processes in the brain and has been suggested to be part of the mechanism underlying improvement in learning and memory in rodents. Moreover, mTOR has been reported to promote neuronal differentiation during neurogenesis. The mTOR pathway can be activated by growth factors such as BDNF through the activation of TrkB receptors, and downstream activation of Akt signaling. Previous results from our group have demonstrated that repeated alpha-linolenic acid treatment per se increases the endogenous expression of mature BDNF protein levels and neurogenesis in the hippocampus of naïve animals, but the intracellular signaling mechanism(s), and the

possible restorative processes activated after a brain insult remains unclear. I want to investigate whether soman–induced neuropathology is associated with changes in the endogenous expression of mature BDNF in the hippocampus and determine a well-established downstream pathway known to be mediated via the activation of TrkB receptors by BDNF, i.e., Akt and mTOR and propose to address whether three doses of LIN treatment modulates these kinases, which in turn may increase neurogenesis and reduce cognitive impairment after soman exposure. Treatment with systemic rapamycin, an inhibitor of mTORC1, will be administrated to further explore whether cognitive performance and neurogenesis are affected after LIN treatment.

Chapter 2: Literature Review

MECHANISMS OF SOMAN TOXICITY

Soman is a potent organophosphorous (OP) nerve agent used in chemical warfare. Soman belongs to the same class of G-series nerve agents as tabun, sarin and cyclosarin. These nerve agents are extremely hazardous due to their superior penetration into the human body and their high toxicity. The dissemination of these agents can cause mass casualties and the individuals that survive exposure can develop long-term disabilities involving both central and peripheral nervous systems [1].

In the past years throughout the world, terrorists have released nerve agents on civilian populations. In the terrorist attacks on Matsumoto [2, 3] and in the Tokyo subway [1, 4], thousands of people were intoxicated and nineteen people died [5]. Last year, over one thousand people were killed in the aftermath of nerve agent use in Syria, including 426 children [6, 7].

The nerve agents soman, sarin and tabun are volatile liquids and persist for a short time in the environment [8]. The vapor/aerosol state enters the body through the respiratory tract and eyes, and the liquid state enters the body through eyes, skin and mouth. In the body and in the environment, nerve agents are degraded by hydrolysis through the loss of fluoride and are converted to their specific corresponding alkyl methylphosphonic acids (AMPAs) for each type of nerve agent [8-10]. The estimated half-life for soman is approximately 60 hours at pH 6.0 and 25ºC [11]. The products from degradation are used as markers for the release of nerve agents. The first product from hydrolysis may undergo further hydrolysis by loss of the O-alkyl group, resulting in the

non-specific methyl phosphonic acid (MPA). This process is very slow in water, but more pronounced when the AMPAs are adsorbed by soil [8, 9].

OP agents exert their effects by inactivating the enzyme acetylcholinesterase (AChE) and causing the accumulation of acetylcholine (ACh) at neuronal synapses and the neuromuscular junction. AChE is the enzyme that catalyzes the hydrolysis of the excitatory neurotransmitter acetylcholine into choline and acetic acid to terminate neurotransmission. Nerve agents inhibit AChE by binding to the active site of AChE, creating a stable, covalent bond. The serine in the catalytic triad (Ser-His-Glu) of the active site of AChE interacts with the phosphate group from soman, providing a stable ester. Over time, a chemical stabilization of the OP-enzyme conjugate, called "aging" irreversibly inactivates AChE. During this process, there is a removal of the alkyl group bond by breaking one of the oxygen –phosphorus bonds on soman, resulting in increased electron sharing between the phosphate group from soman and the serine on the acetylcholinesterase, strengthening the phosphorus-enzyme bond [12]. If the AChE is not "aged", there are some compounds (HI-6, obidoxime, trimedoxine, 2-PAM) containing an oxime group that splits the organophosphorous moiety from the active site resulting in enzyme reactivation [13]. The rate of aging is variable, depending on the toxicity of the nerve agent. For instance, AChE will "age" in only two minutes after interacting with soman [14]. Therefore, it is critical that an oxime be administered immediately after soman exposure to allow some reactivation of AChE [15], an impossible feat during a mass casualty scenario. Also, there are currently no FDA-approved drugs that ameliorate the long-term nerve agent-induced cognitive and behavioral deficits. For these reasons,

more effective therapies are greatly needed to reduce nerve agent-induced neuropathology and the attended long-term deficits.

Inhibition of AChE leads to a rapid increase in ACh levels in synapses in the central nervous system (CNS) and peripheral nervous system (PNS). Accumulation of ACh in the synaptic cleft leads to the overstimulation of ACh receptors, triggering seizure activity. The rapid increase of Ach that occurs early in soman poisoning is known as the cholinergic phase. During this phase seizures can be blocked with muscarinic receptor antagonists when given immediately after nerve agent exposure. If not controlled, continued seizure activity recruits glutamate and possibly other neurotransmitters to propagate and maintain seizures. This is the transitional phase with modulation of cholinergic/non-cholinergic systems. During this phase, seizures cannot be stopped with muscarinic receptor antagonists. Moreover, glutamate can further stimulate the release of ACh contributing to prolongation of the seizures and CNS neurotoxicity. [16, 17]. The initial seizure will trigger a cascade of effects (cellular edema, cytotoxicity, ion imbalance – Ca^{2+} influx - and inflammation) leading to hypoxic-ischemic injury, the major contributor to neuronal death and neuronal loss, after OP poisoning, followed by long-term neurological and behavioral deficits [Figure 1] [18-20]. Follow-up studies have shown that people exposed to nerve agents complain of a plethora of symptoms with long-term neurological and behavioral effects being the most debilitating consequences [18, 21-27].

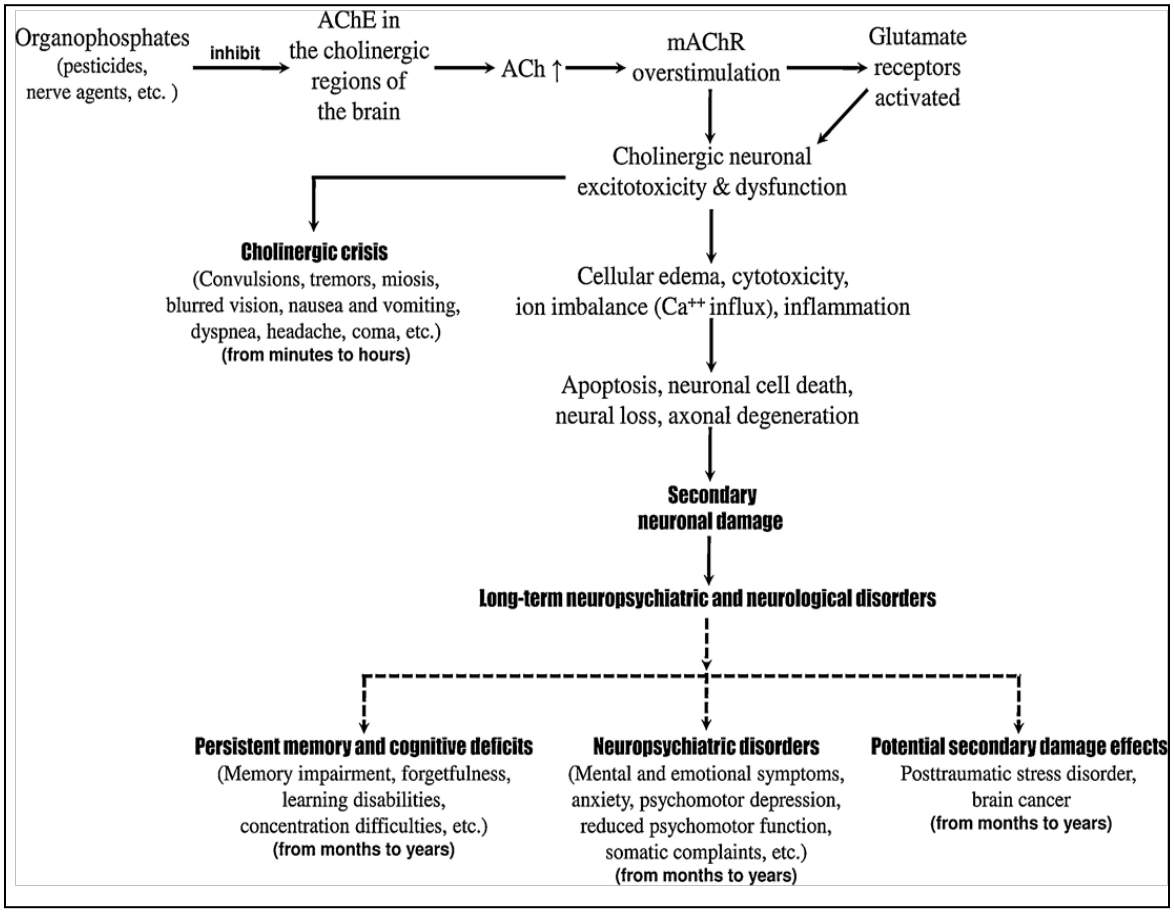

Figure 1. Progression to hypoxic-ischemic neuronal cell death in the brain after OP exposure.
Taken from Chen 2012 [20]

Soman-induced seizures rapidly progress to *status epilepticus* and excessive synaptic accumulation of acetylcholine affects other organ systems beside the brain causing hypersecretions, respiratory distress, tremors, convulsions, coma, and death [16]. However, studies have suggested that seizures induced by nerve agents differ from other epileptic conditions [28] and classical clinical antiepileptic drugs are ineffective against soman-induced seizures with the exception of the benzodiazepines [29].

Despite the cholinergic effects that have been extensively documented in OP toxicity, excessive release and overactivation of glutamate receptors play an important role in OP nerve agent neuropathology. The N-methyl-D-aspartate (NMDA) glutamate receptor subtype has been especially implicated since stimulating this receptor results in a massive influx of calcium through the NMDA receptor channel. This event is known as excitotoxicity and is implicated in the pathogenesis of trauma, ischemia, epilepsy and other neurodegenerative diseases [30]. This stage presumably occurs relatively quickly after seizure activity has commenced and is a predominantly glutamatergic phase of soman-induced seizures. An *in vivo* study has demonstrated increased levels of extracellular glutamate following soman intoxication in the piriform cortex and hippocampus starting within 20 min of seizures [31, 32].

To increase animal survival in research using nerve agents, animal models of OP toxicity receive pharmacological treatment with oximes (HI-6, pralidoxime, obidoxime) to reactivate the AChE, and atropine methyl nitrate, an antagonist of muscarinic receptors. Neither drug crosses the blood-brain barrier. This life-saving treatment is based on the current drug use development for personnel entering areas of nerve agent contamination. To allow standardization of seizures among the experimental groups, a third drug, diazepam, is administered after OP injection of soman to terminate the *status epilepticus*, although recurrent seizures do occur, and diazepam was unable to prevent neuropathology [16, 33, 34]. During the first two to four days after soman-induced seizures, there is a significant body weight loss (up to 20%) regardless of treatment with diazepam [19, 35]. During this time, animals also suffer from dehydration that is counteracted by injection of Lactate Ringer's solution during the first week after soman

exposure. Both in rats and humans, these drug treatments are able to attenuate seizures and reduce peripheral signs of OP toxicity, but are not effective in preventing the brain damage and long-term neurological sequelae.

The brain regions damaged by soman exposure are the piriform cortex, amygdala, prefrontal cortex, hippocampus, caudate/putamen, and thalamus [36]. The histopathological alterations that occurs after OP exposure can last up to at least three months in the affected brain regions [20]. The neuronal degeneration induced by OP agents occurs mainly by necrosis, but apoptosis and hybrid forms have been reported in one study [37]. The necrotic process leading to neuronal death is mediated by NMDA receptor-mediated excitotoxicity after soman poisoning [38, 39].

COGNITIVE IMPAIRMENTS AFTER SOMAN EXPOSURE

Alterations in learning, memory and behavior are deleterious effects of OP intoxication. Soldiers exposed to OP poisoning, as occurred during the 1991 Iraq Gulf War, and civilians in the 1995 Tokyo subway terrorist attack, suffer from neurological and neuropsychiatry disorders – namely, cognitive and memory impairments, as well as deficits in social and emotional function [20]. The amygdala and hippocampus play a well-established role in emotional functioning and learning and memory [40]. Therefore, it is reasonable to predict that cellular damage in these regions after soman exposure could be associated with functional deficits in some behaviors. Behavioral tests are able to access several learning and memory processes involving the hippocampus, cortex and amygdala.

The Morris water maze is a method to assess spatial or place learning that involves the hippocampus circuitry but also the prefrontal cortex, the cingulate cortex,

entorrhinal and perirhinal cortex and neostriatum. The Morris water maze test is widely used as a measure of hippocampal-dependent spatial navigation and reference memory and is relatively immune to motivational differences [41]. On the other hand, fear-based conditioning procedures such as the Pavlovian classical conditioning (the fear conditioning test) and the operant conditioning (the passive or the active avoidance tests) are methods to study learning, memory and also the emotional process that involves the hippocampus, the amygdala, frontal cortex and cingulate cortex [42, 43]. Fear conditioning represents a form of associative learning, e.g., behavioral tasks that promote associations between two stimuli, or between stimuli and responses [44]. In the conditioning task, an innocuous conditioned stimulus (CS) such as a tone, or a light, or an experimental chamber is contingently paired with an aversive unconditioned stimulus (US) such as a mild electric shock (e.g. a footshock). Through CS–US association formation, the CS comes to elicit a fear learning response [45]. These responses can help elucidate mechanisms for memory formation and maintenance and will be fully discussed later in this thesis.

It is also possible to apply other behavioral tests in parallel with memory tests to eliminate the possibility of false positive (or false negative) results. The open field test permits the measurement of spontaneous horizontal (locomotor) activity, which is often used to identify motor and general health performance from a previously administered drug [46].

Experiments with rats and mice injected with soman revealed impairments in several tests such as: Morris water maze [19, 35, 47, 48] passive avoidance [49, 50], active avoidance [51], and fear conditioning [52]. The profound hippocampal injury after

soman exposure has been related to poor performance in the Morris water maze [19]. In the absence of neurodegeneration, no alteration was found in the Morris water task after a low dose of intrahippocampal soman injection indicating that it is the damage that mediates the poor performance [53].

Other behavior alterations such as depression and post-traumatic stress disorder (PTSD) were reported in human studies following exposure to nerve agents [54-57] despite administration of antidotal drugs to prevent seizures [21]. Depression is described as a co-morbidity in epilepsy [58] and is a seriously disabling public health problem of very high prevalence worldwide [59]. Among the behavioral tests available to access depression in rodents, the Porsolt forced swim test is widely used to correlate depressive-like activity [60] and evaluate antidepressant efficacy [60, 61]. Experiments accessing depressive-like behavior have not been yet studied in models of soman exposure in rats. Other organophosphorous compounds that inhibit AChE activity such as malathion have been shown to result in depressive-like behavior as evidenced by an increased immobility time in the forced swim test [62].

MEMORY AND LEARNING IN THE PASSIVE AVOIDANCE TEST

Memory and learning is described by Stuchlik, [63], where "memory refers to a capability of virtually any animal to encode, store and retrieve information, to guide behavioral output" and "learning is viewed as acquisition or encoding the information to memory". The activity in the hippocampus is thought to be a major brain region involved for the formation of a memory, but other regions such as the amygdala has demonstrated to modulate memory consolidation after learning has taken place [64].

The passive avoidance test as the Pavlovian contextual fear conditioning test is believed to be based on hippocampus-dependent contextual memory, which is associated with the place and the event, and the amygdala-dependent emotional memory.

Extensive neuronal depletion in the dorsal hippocampus of female rats disrupt memory in the passive avoidance test [65], and inhibition of protein synthesis using the protein synthesis inhibitor anisomycin by intrahippocampal infusion into the dorsal hippocampus impairs retention test performance [66].

Although the passive avoidance test shares several similarity with the Pavlovian fear conditioning test, the role of the amygdala to modulate memory may differ between these two tests (for review see [64, 67]. Because the amygdala and hippocampus are two brain regions that show profound damage in soman-exposed animals [36], the passive avoidance task was chosen to test hippocampus and amygdala function [68, 69].

In the passive (or inhibitory) avoidance test, memory is acquired through instrumental or operant conditioning in which the presentation of an aversive stimulus is associated with a previous neutral stimulus so that the animal avoids the neutral stimulus [45]. The approach consists of a two-compartment chamber (light and dark) in which the rat is first placed in the light compartment and learns to avoid entering the preferred dark compartment after receiving a single foot shock (acquisition/training/conditioning phase) [46]. The rat is next returned to its home cage, where the memory is consolidated [70], a process by which the newly acquired memories are stabilized into long-lasting memories [64]. The retention or memory test is assessed at different time points (test phases) after training and is measured by the latency in seconds to enter the dark side [46, 68]. This approach has been useful to understand the molecular events that enhance or inhibit

learning and memory and is critical for evaluating new compounds for the treatment of memory disorders.

NEUROGENESIS AND BEHAVIOR

It was suggested recently, that a reduction in neurogenesis in the hippocampus causes behavior alterations [71]. Over the past decade, it has become apparent that new neurons are still formed in particular regions of the adult brain, but their role in neurodegenerative disorders is not clear. Detection of neurogenesis has been made possible by combining BrdU (5'-bromo-2'-deoxyuridine), a thymidine analogue that is incorporated into DNA undergoing replication, with cell-specific proteins present in the newly generated neurons at different stages of development [72].

Neurogenesis occurs in at least two regions of the adult mammalian brain: the subventricular zone (SVZ) in the lining of the lateral ventricles, and the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus. Neurogenesis has a particular importance in the hippocampus because this brain region is a critical area in the brain involved in learning, memory and mood. The hippocampal DG harbors progenitor cells located in the SGZ which is a thin band of tissue adjacent to the innermost layer of granule neurons [73, 74]. The neural progenitor cells located at SGZ give rise to dentate granule cells (DGCs) in the granule cell layer (GCL) where they differentiate into neurons and mature over several weeks [Figure 2] [75]. As they mature, a fraction of those newly generated neurons become integrated and form synapses, glutamatergic input from the entorhinal cortex and output to pyramidal cells in the CA3, in a functional hippocampal network [76]. These processes are modulated both positively and negatively

by neurotransmitters, hormones, neurotrophic factors, pharmacological agents and environmental factors [Figure 2] [77-80]. Brain-derived neurotrophic factor (BDNF) is one of the neurotrophic factors that regulates hippocampal neurogenesis, and will be fully discussed later in this thesis.

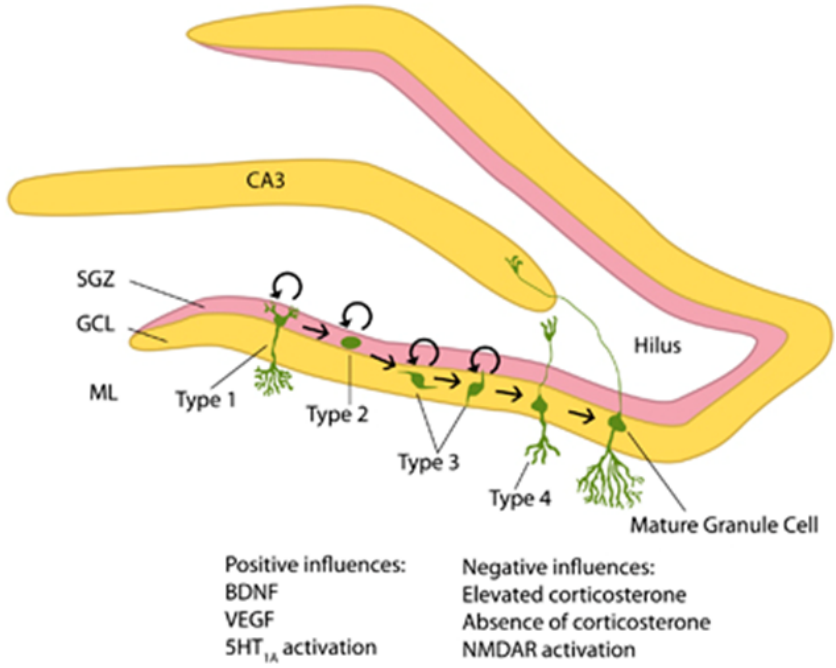

Figure 2. Neurogenesis in the adult hippocampus and examples of positive and negative influences.
Type-1 cells (radial-glia-like stem cells) in the subgranular zone divide asymmetrically, maintaining their population while producing Type-2 daughter cells (neural progenitor cells). These continue to divide symmetrically as they mature into Type-3 cells (neuroblasts) and migrate into the granule cell layer. Type-4 cells, which have ceased mitosis, extend axons toward the CA3, leading to the development of mature granule cells that integrate with the mossy fiber pathway. SGZ, subgranular zone; GCL, granule cell layer; ML, molecular layer. Taken from Hanson et al 2011 [81]

The role of neurogenesis after brain damage resulting from seizures is complex and studies in OP models are limited. Also, differences in genetic variation and traits influence adult neurogenesis [82, 83]. It is worth noting that the duration of seizures

determine the degree of neuropathology. Evidence has suggested that seizure-induced brain damage results in impairment in axonal sprouting and aberrant neurogenesis. In models of *status epilepticus* (SE) with systemic pilocarpine or kainic acid injection, neurogenesis of granule cell of DG increases within the first 4 weeks of the insult. In those animals, new neurons produced after *status epilepticus* showed extensive mossy fiber sprouting when examined ten weeks after *status epilepticus* [84], whereas newborn neurons produced seven weeks prior to status developed normally making a critical case for the timing of the birth of new neurons in the DG. On the other hand, chronic epilepsy reduces neurogenesis in humans [85].

In studies with soman, mice were injected with soman followed by an oxime and atropine in the absence of diazepam. In those studies, the DG cells that were marked only with the proliferation–marker bromodeoxyuridine (BrdU) showed the following changes: a decrease after the first day, an increase at the third day, no changes between day 8 and 30 compared to the control, and a marked reduction in BrdU-positive cells 90 days after the insult, with a very low number of mature neuronal cells double marked with NeuN at the 34th day [86]. In another study, double-labeling with BrdU and the immature neuronal marker doublecortin (DCX), showed a decrease in neurogenesis on the 28th day after soman exposure with an associated spatial learning impairment [35].

The participation of newly born neurons in hippocampal processing has been recently discussed in reports showing that changes in neurogenesis are associated with learning performance in hippocampus-dependent learning tasks [87]. Reduced hippocampal neurogenesis has been shown to result in deficits in contextual fear conditioning (Imayoshi et al., 2008; Saxe et al., 2006; Warner-Schmidt et al., 2008;

Winocur et al., 2006; Drew et al., 2010; Denny et al., 2011), spatial long-term memory (Snyder et al., 2001), trace memories (Shors et al., 2001; Shors et al.,2002), spatial relational memory (Dupret et al., 2008), and pattern separation (Clelland et al., 2009). Reduced neurogenesis has also been implicated in mood disorders, such as depression, but recent studies have raised several controversies [88]. Recent studies using the passive avoidance test have now demonstrated that baseline neurogenesis is required for hippocampal learning and long-term memory formation. Reduced neurogenesis through rapid x-ray ablation causes impairment in performance of the passive avoidance task [89, 90].

Conversely, a variety of studies have consistently demonstrated that enhanced neurogenesis plays an important role in exerting the therapeutic efficacy of anti-depressant agents [82] and its potential to affect contextual-memory systems has been increasingly recognized [91-94]. Consistent with experimental studies, a computational approach has been able to draw a model to understand how hippocampal networks are likely to be selected for encoding information (Figure 3). In the hypothesis of how new neurons affect learning and memory, the new neurons are *added* to the DG network, instead of replacing existing neurons. This addition could effectively encode and retrieve new memories in the network without interfering with old memories [95]. However, new research has proposed that the continuous integration of new neurons may affect memories already stored in these circuits by competing with existing cells for inputs and outputs when examined over longer times [96].

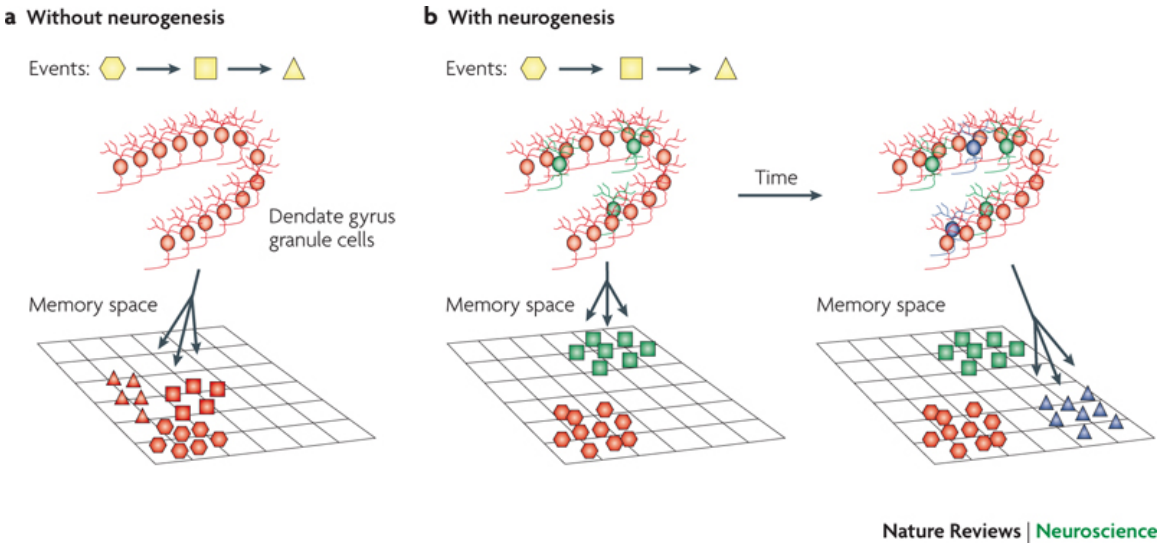

Figure 3. Computational theories of neurogenesis.
A. Without neurogenesis, new events (represented by different shapes) are limited by the set of sparse 'codes' (combinations of active neurons) provided by mature granule cells in the dentate gyrus. This can lead to the dentate gyrus not having the flexibility to encode new memories well [97, 98] or to interference between memories formed in the hippocampus (shown as a cluster of memories in a projection of the high-dimension hippocampal 'memory space') [99]. B. New neurons (shown in green) provide new sparse codes for encoding new information, while older memories are preserved because they are represented by older neurons (shown in red). This can facilitate the formation of new memories while avoiding catastrophic interference, saving older memories (shown in the left panel as two separate clusters of memories in a projection of the high-dimension hippocampal memory space) [97-99]. The three-way arrow indicates that new neurons can change how memories are encoded in the hippocampal network. Neurons born at different times (shown in green and blue in the right panel) represent different inputs, and the sparse codes generated at a particular time are clustered together (active neurons in a population are similar in composition to one another), separately from sparse codes that were generated at a different time, essentially encoding time into new memories [100-102]. Taken from Deng et al 2010 [95].

ALPHA-LINOLENIC ACID AS A POTENTIAL THERAPY

In this study, we employ soman as a model of OP-induced neurotoxicity and cognitive impairment, to examine the neuroprotective efficacy of alpha-linolenic acid (LIN, 18:3n-3); an essential omega-3 polyunsaturated fatty acid (PUFA). LIN is an essential PUFA that must be obtained from the diet and is found in green leaves, seed oil

(flaxseeds), beans and walnuts. Flaxseed oil is particularly rich in LIN (>50%) and is a nutraceutical. LIN is essential because humans are unable to insert a double bond at the n-3 position of a PUFA. LIN and other omega-3 (or n-3) PUFA family members have their first double bond three carbons from the omega end.

In humans as well as rats, the major route of metabolism of LIN after oral ingestion is through beta-oxidation, with most LIN metabolized to CO_2 or acetate for *de novo* lipid synthesis and ketones bodies (for review see: [103]. More studies need to be done to clarify the disposition of LIN by the intravenous route, which can rapidly increase plasma LIN levels and consequently increase tissue uptake [104].

A second metabolic pathway for LIN is the conversion to its respective long-chain PUFA products, EPA (ecoisapentaenoic acid, 20:5n-3) and DHA (docosahexaenoic acid, 22:6n-3), through a series of desaturation and elongation steps. The process of desaturation and elongation in the liver is the most important in terms of the supply of LIN metabolites to other tissues. Brain and other organs can convert LIN to DHA, however when compared with the liver, the rate of brain DHA synthesis in the rat is very limited [105, 106]. This pathway involves seven different steps where LIN is first converted to EPA and later DHA with most of the reactions occurring in the endoplasmatic reticulum [107].

Although several studies have demonstrated the ability of rats and humans to synthesize EPA and DHA from LIN, the overall conversion is very limited, ~5% to EPA, ~1% to DHA in humans and ~6% in rats (for review see: [[108]]), which raises the possibility that LIN exerts actions of its own. In an *in vitro* model of glutamate excitotoxicity, neuronal death is effectively inhibited by LIN and other PUFAs, but not

by saturated fatty acids in mouse cerebellar neurons [109]. Moreover, LIN treatment increases BDNF protein levels in neuronal hippocampal cultures and in neural stem cells (NSC) followed by increased neuronal proliferation [110], suggesting some direct effects of LIN-induced neuroprotection in the brain. Although other PUFAs also exhibited neuroprotective properties, LIN produced the most efficacious and reproducible effects. In a well-established model of epilepsy induced by kainic acid, LIN treatment, but not other PUFAs, was able to almost completely abolish neuronal cell death in the hippocampal CA1 and CA3 subfields [109]. LIN treatment has also been demonstrated to be a potent neuroprotective agent in several models of global and focal ischemia [109, 111-113]. Additionally, recent findings from our laboratory demonstrated that a single injection of LIN is able to reduce neuronal cell death induced by soman, without stopping seizures [36]. This finding suggests that LIN acts by preventing the secondary neuronal damage – progressive neuronal cell death, neural loss and axonal degeneration [20] - triggered after initial soman-induced seizures, which could reduce the attended cognitive impairments.

ALPHA-LINOLENIC ACID AND BDNF EFFECTS IN THE BRAIN

The particular mechanisms underlying the neuroprotective effects of LIN are not clear. *In vivo* subchronic LIN treatment administered on day 1, 3 and 7 by intravenous injection in naïve mice increases the endogenous expression of mature BDNF in the cortex and hippocampus, and this increase was associated with an enhancement in neurogenesis, synaptogenesis and synaptic function in mice confirming the *in vitro* studies [110]. In the same study, LIN demonstrated significant antidepressant-like efficacy. My hypothesis is that the LIN-induced increase of mature BDNF in the

hippocampus activates its receptor, tropomyosin related kinase B receptor (TrkB), activating downstream effectors to increase neuronal survival, synaptic plasticity and neurogenesis, which is associated with an enhancement in functional outcome (e.g., antidepressant/learning and memory) after brain injury. One issue that will still remain unresolved is the source of BDNF after LIN treatment.

BDNF is a member of the neurotrophin family that also includes nerve growth factor (NGF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4/5) [114]. The mouse and rat BDNF gene has eight 5' untranslated exons (exons I-VII) that are controlled by distinct activity-dependent and tissue-specific promoters, and one 3' exon (exon IX) that encodes the BDNF polypeptide [Figure 4A]. The complex structure of the BDNF promoter allows alternative splicing and polyadenylation induced by different cell types or physiological conditions resulting in differential transcriptional regulation of the expression of BDNF transcripts [115, 116]. The mechanisms of BDNF gene regulation by different promoters are not well-understood. Cumulative evidence has suggested that promoters I and IV are responsive to neuronal activity (e.g., membrane depolarization, glutamate release, kainate-induced seizure and sensory experience). Regulatory elements located within promoter II of the BDNF gene is related to brain specificity of BDNF expression by repressing BDNF transcription in non-neuronal cells. And epigenetic mechanisms (e.g., DNA methylation and histone deacetylation) have been associated with repression or activation of the promoter IV and VI in the BDNF gene [Figure 4B] [115-119].

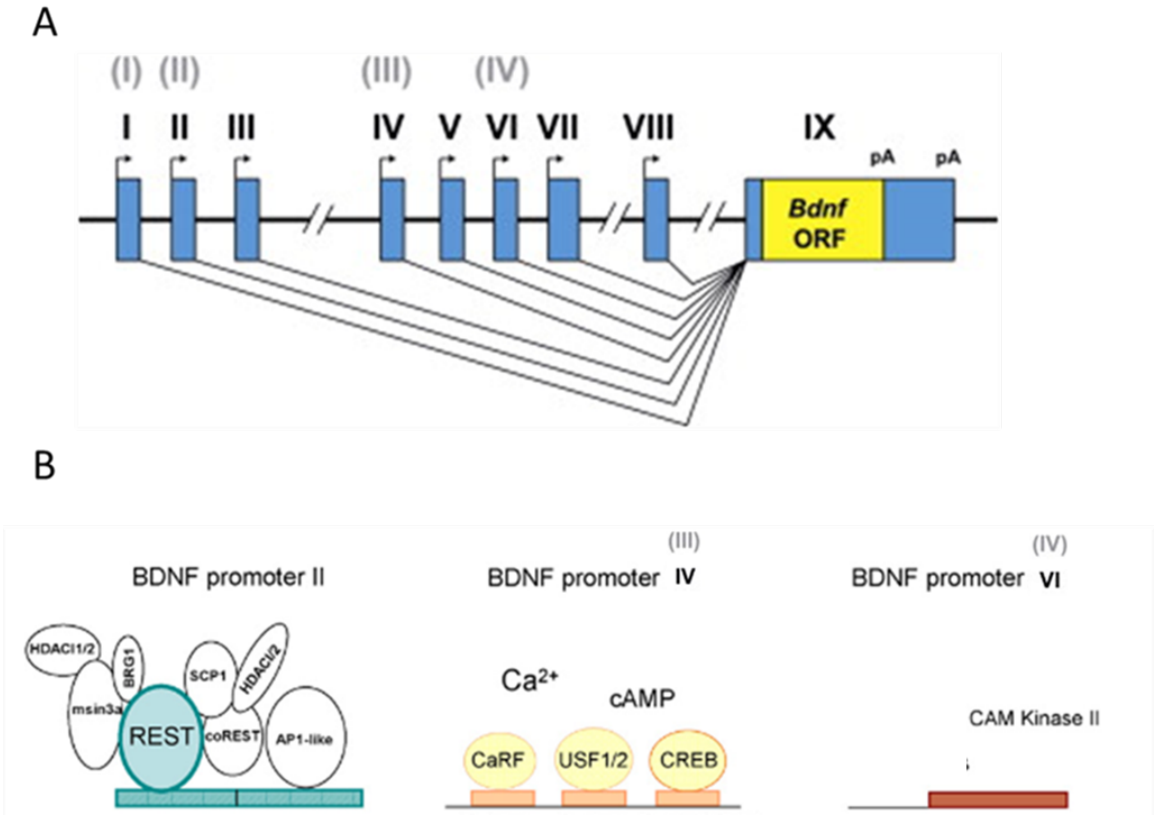

Figure 4. Schematic Representation of the BDNF Gene
A. BDNF consists of at least eight distinct promoters (I–VIII) that initiate the transcription of multiple distinct BDNF mRNAs, each of which contains an alternative 5′ exon spliced to a common coding exon (exon IX) that employs either of two polyadenylation sites (pA), so as to generate one short splice variant and one long splice variant. Additional exons have only been recently identified; BDNF exons in gray (above) is the old nomenclature previously identified by Timmusk et al [120]. For example, the current exon IV was previously referred to as exon III. ORF : open reading frame. Modified from Greer and Greenberg 2008 [117].
B. *Left*: REST (RE1 silencing transcription factor protein) repressor complex (blue and white circles) binds to silencer elements within the promoter II and represses BDNF transcription in non-neuronal cells. *Centre:* Promoter IV is regulated by transcription factors responsive to Ca^{2+} and cAMP (cyclic AMP) levels such as CaRF (Ca^{2+}-response factor), USF (upstream stimulatory factor 1/2) , CREB (cAMP response element [CRE]-binding protein) and nuclear factor kappaB (not included in the figure). *Right:* Epigenetic regulation of chromatin structure modulates protein action on BDNF exon VI promoter. CaMKII (Ca^{2+}/calmodulin kinase II). Modified from Zuccato and Cattaneo 2007 [118].

In addition to transcriptional regulation, BDNF expression is also controlled at the post-transcriptional level. BDNF is first synthesized as a precursor protein known as

prepro-BDNF that is cleaved into pro-BDNF (32 kDa), which can then be further cleaved into mature BDNF (14 kDa) by several mechanisms [115, 119]. Pro-BDNF binds to the low-affinity neurotrophin receptor p75 that is believed to be involved in apoptosis [121]. Mature BDNF signals through the high-affinity TrkB receptor and is important for maintaining survival, synaptic plasticity and neurogenesis in the adult CNS. Both BDNF and TrkB are widely expressed in the hippocampus and cerebral cortex [122, 123]. At the cellular level, studies have indicated that BDNF is mainly present in neurons. Glial cells also express BDNF but a function has not been defined. It has been proposed that glial cells express the truncated form of TrkB receptor that mediates BDNF internalization and storage under endogenous or exogenous stimuli [118].

Stimulation of BDNF production enhances hippocampal proliferation [124] and TrkB is required for the morphological maturation and survival of new neurons [125]. Genetic deletion of a single allele of the BDNF gene (heterozygous BDNF knockout mice) or conditional deletion of the TrkB gene in neural progenitor cells of mice impairs proliferation and neurogenesis in the DG of the hippocampus, and leads to loss of antidepressant efficacy [126-128]. Additional studies have also linked increased BDNF levels and TrkB activation to antidepressant effects [129-131]. However, deletion of a single allele of the BDNF gene or conditional deletion of TrkB from the DG in the hippocampus does not result in depression-like behavior [126, 127]. Taken together, these data indicate that an increase of BDNF levels and subsequent TrkB activation resulting in the activation of downstream signaling pathways may be mediating the biological responses involved in neuroprotection and behavior.

BDNF/TRKB DOWNSTREAM SIGNALING PATHWAY

After activation by BDNF, the TrkB receptor monomers dimerize, which increases the catalytic activity of the intracellular domain of the intrinsic tyrosine kinase. This enables the phosphorylation of tyrosine residues inside the activation loop and subsequently the autophosphorylation of tyrosine residues situated outside of the activation loop. The next step is the activation and recruitment of partner proteins and adaptors that lead to the activation of three main intracellular signaling pathways: 1) the phospholipase C γ (PLC γ) pathway, which leads to activation of protein kinase C; 2) the mitogen-activated protein kinase (MAPK) or extracellular signal-regulated kinase (ERK) pathway, which activates several downstream effectors; and 3) the phosphatidylinositol 3-kinase (PI3K) pathway, that activates the serine/threonine kinase Akt. Both MAPK and PI3K play crucial roles in neuronal survival, protein-synthesis dependent plasticity and neurogenesis.

The MAPKs are a specific class of serine/threonine kinases that respond to extracellular signals such as growth factors, mitogens, cellular stress and mediate proliferation, differentiation, cell survival, learning and memory and cognition in mammalian cells [132]. There are four distinct groups of MAPKs within mammalian cells: the extracellular signal-regulated kinases (ERKs), the c-jun N-terminal kinases (JNKs), the atypical MAPKs (ERK3, ERK5, and ERK8), and the p38 MAPK. Several investigations have indicated that ERK promotes cell survival and proliferation, whereas JNK and p38 MAPK induces apoptosis. ERK has a threonine-glutamic acid-tyrosine (Thr-Glu-Tyr) motif and is divided into two subtypes: ERK1 and ERK2. Activation of ERK by neurotrophins stimulates RAS, which activates MAPKKK (raf). Raf

heterodimerizes (c-raf/b-raf) and activates MAPKK (Mek 1/2) which in turn activates MAPK (ERK1/2) [123, 132, 133]. Strong activation of the RAS-ERK pathway cross-activates the PI3K-Akt pathway and promote mTORC1 activity [Figure 5] [134, 135].

The second major intracellular signaling pathway activated by BDNF is the PI3K-Akt pathway. Akt is a serine/threonine protein kinase and a major downstream target of the PI3K pathway. This pathway plays a major role in neuronal survival, and recently has been associated with synaptic plasticity and antidepressant behavior [136]. Low expression of Akt has been related to neurodegeneration, whereas the increased activation of Akt is neuroprotective [137]. The phosphorylation of Ser^{473} and Thr^{308} of Akt fully activates Akt and leads to the activation of a diverse number of protein substrates containing the consensus sequence of RXRXXS/T [138, 139]. The R residues are Arginine, X represents any amino acid, S represents Serine and T is threonine [139]. Akt phosphorylation at Ser^{473} is associated with neuroprotection after seizure-induced neuronal cell death [140]. Akt phosphorylates and inactivates the pro-apoptotic protein Bcl-2-associated death promoter (BAD) and prevents the processing of proapoptotic proteins. The PI3K-Akt pathway has also been shown to activate NFκB survival signaling or inhibits the JNK/p38 apoptotic signaling. Besides survival, the PI3K-Akt pathway is also involved in proliferation. Phosphorylation and inhibition of GSK3 by Akt enhance the stability of multiple downstream targets impinging on cell-cycle regulation. Moreover, the PI3K-Akt pathway plays a key role in controlling cell survival, division and metabolism through the activation of mTOR complex 1 (mTORC1), which is regulated by both nutrients and growth factor signaling [Figure 5] [134, 139].

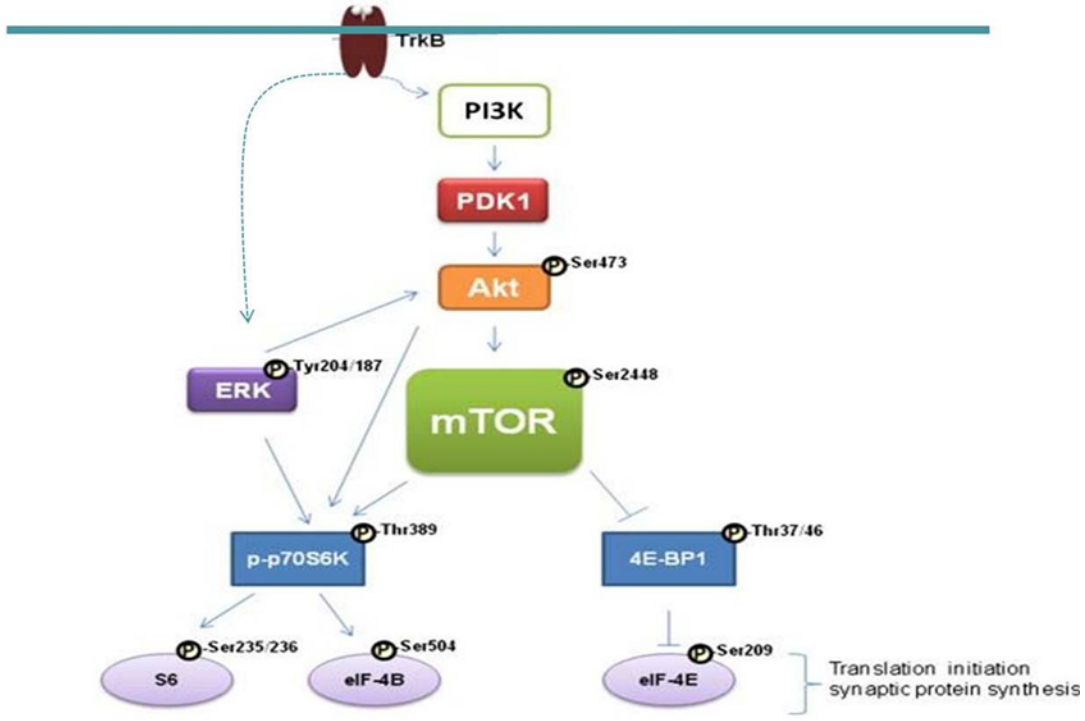

Figure 5. Simplified diagram illustrating the signal transduction pathway leading to mTOR activation and downstream effectors.
Modified from Chandran et al 2013 [134].

Mammalian target of rapamycin is a large (~289 kDa) serine-threonine kinase that exists in two distinct heteromeric protein complexes referred to as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). The mTORC1 is sensitive to the selective inhibitor rapamycin and it is activated by phosphorylation of serine^{2448} in the canonical PI3K- Akt pathway after growth factor stimulation [Figure 5]. However, additional signaling cascades also activate mTOR [141]. Rapamycin does not directly inhibit mTOR kinase activity; instead, it binds to the immunophilin FK506-binding protein 1A, (FKBP12) and disrupts the mTOR-RAPTOR interaction, e.g. the complex of mTORC1 [Figure 6]. By contrast, mTORC2 is resistant to rapamycin, and recent studies have

suggested that mTORC2 may phosphorylate Akt at serine^{473} [142, 143] but its mechanisms in brain plasticity and neurogenesis are not well understood.

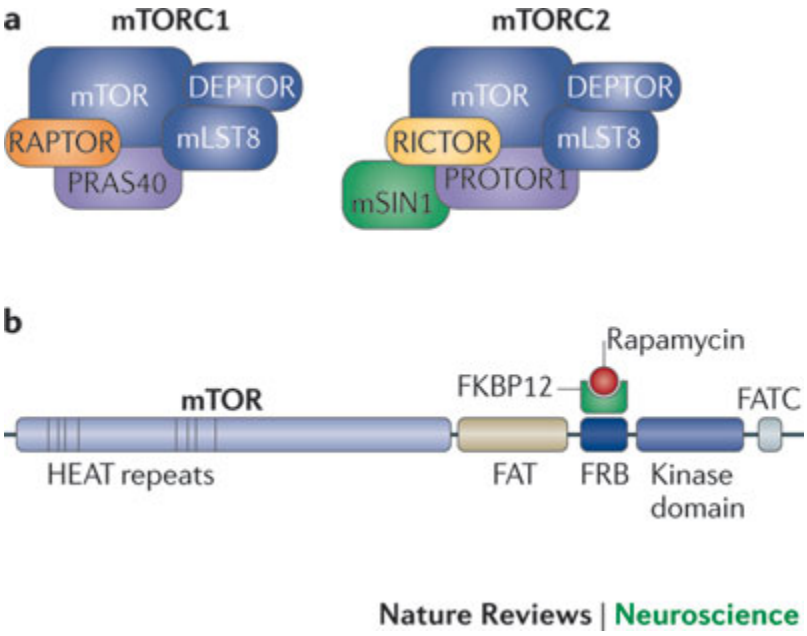

Figure 6. The two distinct mTOR-containing complexes.
A. The mTORC1 contains RAPTOR (regulatory-associated protein of mTOR) and PRAS40 (a 40 kDa proline-rich AKT substrate). mTORC2 contains RICTOR (rapamycin-insensitive companion of mTOR), mSIN1 (mammalian stress-activated MAP kinase-interacting protein 1; also known as MAPKAP1) and PROTOR1 (protein observed with RICTOR1). RAPTOR acts as a scaffolding protein linking mTOR kinase with mTORC1 substrates, thereby promoting mTORC1 signaling. B. Rapamycin associates with its intracellular receptor, FKBP12 (FK506-binding protein of 12 kDa; also known as PPIase FKBP1A), and the resulting complex interacts with the FRB (FKBP12–rapamycin binding) domain located in the carboxyl terminus of mTOR175. Binding of rapamycin–FKBP12 to the FRB domain of mTOR disrupts the association of mTOR to the mTORC1-specific component RAPTOR and thus uncouples mTORC1 from its substrates, thereby blocking mTORC1 signaling. Taken from Bove et al 2011[144]

THE ROLE OF MTORC1 IN NEUROLOGICAL DISORDERS

Treatments that activate Akt and mTOR are known to mediate neuroprotective effects against ischemic brain injury [145], and contribute to recovery of motor function after spinal cord injury [146].

Activation of the Akt/ERK/mTOR signaling pathways in the brain mediates protein-dependent synaptic plasticity [147], neural differentiation and proliferation during neurogenesis [148-150], and has been proposed to be part of the mechanism underlying antidepressant effects [133]. The antidepressant effect tested in naive rats is abolished when animals are treated with either rapamycin or with the PI3K inhibitor (LY294002) or the ERK inhibitor (U0126), an effect possibly related to concomitant inhibition of synaptogenesis [136].

However, hyperactive mTOR has been proposed to play a critical role in the development of epilepsy as inhibition of mTOR has antiepileptogenic properties in some models of epilepsy [151-153]. On the other hand, chronic inhibition of mTOR has been reported to induce depressant effects in a rat model of absence epilepsy [154]. In a recent extensive review of the effects of mTOR on epilepsy, Russo et al. [155] conclude that the activation status of mTOR is "complex and variable" and "might be limited to certain types of epilepsy and epileptogenic process", suggesting that the specific type and nature of the brain insult may contribute to the degree of mTOR activation.

The activation of mTOR signaling by neurotrophins increases the translation of synaptic proteins, which is essential for synaptic plasticity, by two mechanisms [Figure 5]. First, mTORC1 phosphorylates and inactivates the eIF4E-binding protein (4E-BPs), facilitating translation initiation by releasing the inhibition of eukaryotic initiation factor 4E (eIF4E), which is a crucial initiation factor in cap-dependent translation. The

association of 4E-BPs with eIF-4E inhibits the ability of eIF-4E to associate with eIF-4G and initiate translation. Second, it activates p70S6 kinase, an enzyme that controls translation at a number of levels, including synthesis of the S6 ribosomal subunit, phosphorylation of RNA helicase cofactor eIF4A, and inhibition of eukaryotic elongation factor 2 (eEF2) kinase [156]. The p70S6 kinase and 4E-BP are also regulated by MAPK/ERK to control protein-synthesis dependent plasticity [Figure 5] [157], and p70S6 can further phosphorylate mTOR setting up an autoregulatory mechanism. Protein-dependent synaptic plasticity strengthens the neuronal connection and would be expected to regulate memory storage in the brain. On the other hand, excessive protein synthesis results in behavioral deficits, instead of improving neuroplasticity, suggesting a temporal window to where mTOR should be carefully modulated [156].

New research has found that rather than just increasing translation of new proteins for synaptic plasticity, mTORC1 activation leads to the induction of genes encoding the enzymes of glycolysis, pentose phosphate pathway and lipid sterol biosynthesis, generating the building blocks for anabolic cell growth [158]. This process could help the development and maturation of new neurons in adult neurogenesis and also cellular repair. The newly generated neurons integrate multiple signals into local circuitry of the hippocampus and several lines of evidence have revealed the importance of these newborn neurons in the acquisition and retention of memories [93, 94, 100, 159] and mood control [160]. On the other hand, mTOR dysregulation could be responsible for imbalanced homeostasis, leading to the development of pathology. Thus, future directions and therapeutic strategy should work towards fine-tuning the mTOR signaling pathway to exert neuroprotective effects in CNS.

Altogether, these observations suggest a central pathway for the LIN-induced increase in mature BDNF protein levels to enhance neuronal survival and neurogenesis in order to ameliorate behavioral deficits after soman-induced neuronal damage and cognitive impairment in rodents.

Chapter 3: Intravenous administration of three doses of alpha-linolenic acid attenuates soman-induced neuropathology and improves functional outcome

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Key words: rat, alpha-linolenic acid, soman, behavior, neuroprotection, passive avoidance, Porsolt forced swim test

Running title: Survival-promoting effects of alpha-linolenic acid against soman

ABSTRACT

Exposure to nerve agents results in severe seizures or status epilepticus caused by the inhibition of acetylcholinesterase, a critical enzyme that breaks down acetylcholine to terminate neurotransmission. Prolonged seizures cause brain damage and can lead to long-term consequences. Current countermeasures are only modestly effective against brain damage, supporting interest in the evaluation of new and efficacious therapies. Alpha-Linolenic acid (LIN) is an essential omega-3 fatty acid that plays a key role in normal health and has been demonstrated to exert neuroprotective efficacy in several models of brain injury. Previous work showed that a single intravenous dose of alpha-linolenic acid administered before or after soman significantly protected against soman-induced brain damage when analyzed 24h after exposure. Here, we show that administration of three doses of alpha-linolenic acid, injected intravenously over a seven day period after soman, significantly improved motor performance on the rotarod, enhanced memory retention, exerted anti-depressant-like activity and increased animal survival. This dosing schedule significantly reduced soman-induced neuronal degeneration in four major vulnerable brain regions up to twenty-one days. In contrast, administration of three doses of alpha-linolenic acid injected intravenously over a seven day period prior to soman exposure did not provide long-lasting neuroprotection or improve functional outcome. Taken together, alpha-linolenic acid reduces the profound behavioral deficits induced by soman, increases animal survival and provides long-lasting neuroprotection only when given after soman, indicating that the timing of alpha-linolenic acid treatment relative to soman exposure is a crucial factor.

INTRODUCTION

Organophosphorous (OP) nerve agents such as soman (O-1,2,2-trimethylpropylmethyl-phosphono-fluoridate), are extremely hazardous compounds leading to generalized seizures, *status epilepticus* (SE) and death [16]. Prolonged seizures can lead to neuronal loss and neurodegeneration, which likely contribute to the development of a variety of cognitive impairments, including deficits in learning and memory and neuropsychiatric disorders such as depression. Nerve agents have been used in terrorist attacks on civilian populations [1, 2, 4, 5] resulting in mass casualties as recently reported in Syria [6, 7]. People exposed to nerve agents develop a plethora of symptoms with long-term neurological and behavioral deficits being the most debilitating consequences [18, 21-23, 25-27].

The mechanism of OP action is through the inactivation of acetylcholinesterase (AChE), the enzyme that catalyzes the hydrolysis of the excitatory amino acid acetylcholine (ACh) into choline and acetic acid to terminate neurotransmission. In the brain, accumulation of Ach at neuronal synapses overstimulates Ach receptors, primarily muscarinic receptors (mAChR), triggering seizure activity in the piriform cortex, septal area, hippocampus, entorhinal cortex and amygdala [161-164]. After the initial cholinergic overstimulation, excessive release of glutamate maintains and propagates seizures leading to hypoxic-ischemic injury and N-methyl-D-aspartate (NMDA) receptor-mediated neuronal cell death [16, 17, 29, 165-167]. The piriform cortex, amygdala and hippocampus, demonstrate the most severe neuropathology after OP exposure [163, 168-172], and these brain regions likely play a pivotal role in the long-term cognitive and behavioral deficits reported in humans and rodents after OP intoxication.

Learning and memory deficits were found in rats exposed to soman assessed by the Morris water maze test [19, 35, 47, 48] passive avoidance [49, 50], active avoidance [51], and fear conditioning [52]. Years after exposure to sarin, victims of the Tokyo subway attack presented with significant declines in psychomotor and memory functions [24, 25], as well as structural changes in the amygdala and cingulate cortex that are associated with a diagnosis of PTSD [173, 174]. Behavior alterations such as depression and PTSD were reported in human studies following exposure to nerve agents [54, 56, 57, 173-175], despite administration of antidotal drugs to promote survival and stop the nerve agent-induced seizures (Bajgar et al., 2004). These results demonstrate the need for more efficacious therapies to protect the brain against OP-induced neuropathology and long-term consequences.

Different therapeutic strategies have emerged over the years as methods to protect the brain against various toxic insults. Both pretreatment and post-treatment therapeutic interventions have shown efficacy against N-methyl-D-aspartate (NMDA) receptor-mediated excitotoxicity *in vivo* [176-180]. Recently, we showed that administration of a single dose of alpha-linolenic acid (LIN) three days prior to (pretreatment) or thirty minutes after (post-treatment) soman exposure reduced neuronal degeneration in vulnerable brain regions when neuronal degenerating neurons were quantified twenty-four hours after soman [36]. However, the long-term effects of LIN against soman-induced neuropathology are unknown.

LIN is an essential omega-3 polyunsaturated fatty acid (PUFA) found in green leaves, seed oil (flaxseeds), beans and walnuts and can be purchased over-the-counter. LIN plays an important role in brain function and protection [181] with no side effects

reported [182, 183]. LIN is a precursor of the long-chain PUFAs, ecoisapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Although humans and rats have the ability to metabolize LIN to form EPA and DHA, the overall conversion is very limited in humans [108] and in rats [184] raising the possibility that LIN exerts actions of its own. Glutamate-mediated excitotoxicity via overactivation of NMDA receptors is effectively inhibited by LIN in cultured hippocampal neurons [110], but not by saturated fatty acids in mouse cerebellar neurons [109]. Although other PUFAs also exhibited neuroprotective properties *in vivo*, LIN produced the most efficacious and reproducible effect [109]. In a well-established model of epilepsy induced by kainic acid, LIN treatment, but not other PUFAs or saturated fatty acids, was able to almost completely abolish neuronal cell death in the hippocampal CA1 and CA3 subfields [109]. LIN treatment has also been demonstrated to be a potent neuroprotective agent in several models of global and focal ischemia [110, 112, 113, 179]

Our recent results demonstrated that administration of a single intravenous dose of LIN administered either before or after soman significantly protected against soman-induced neuropathology without altering seizure latency or severity [36]. This finding suggested that LIN may be capable of preventing the primary as well as the secondary neuronal damage triggered by soman, which in turn could reduce the attendant cognitive impairment. In naive mice, administration of LIN at 30 min, 3 days and 7 days after initial injection was demonstrated to increase the levels of mature brain-derived neurotrophic factor (mBDNF) in the cortex and hippocampus and is associated with increased synaptogenesis, neurogenesis, and synaptic function. In addition, subchronic LIN treatment exerted an antidepressant-like activity [110].

The long-term goal of our work is to develop highly efficacious therapies to protect the brain against nerve agent-induced neuropathology and its long-term consequences. Here, we extend our work using soman as a model of organophosphorous-induced neuropathology to examine the neuroprotective efficacy of subchronic administration of alpha-linolenic acid (LIN, 18:3n-3) and its effect on behavior measures. In this study, we investigate whether administration of three injections of LIN (500 nmol/kg) administered intravenously either prior to or after soman increases animal survival, provides long-lasting neuronal survival and improves functional outcome in Sprague-Dawley male rats.

MATERIALS AND METHODS

Animals

Experiments were performed on male Sprague-Dawley rats weighing 300–350 g (Taconic Farms, Germantown, NY). The animals were maintained on a 12 h light/dark cycle and given food and water ad libitum. The animals were acclimatized for one week prior to drug treatment or surgery. The experimental protocol was approved by the Animal Care and Use Committees at the Uniformed Services University of the Health Sciences and United States Army Medical Research Institute of Chemical Defense and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act of 1966 (P.L. 89-544), as amended. All efforts were made to minimize the number of animals used.

Placement of external jugular vein catheter (JVC)

All surgeries were performed under aseptic conditions. Anesthetized rats (isoflurane 2.5 –

3%, with oxygen) were placed in the dorsal recumbent position. Briefly, a 1.5–2 cm long incision was made in the ventrolateral aspect of the neck, parallel and approximately 0.5 cm lateral to midline. The external jugular vein was dissected free of surrounding tissue and stabilized with a proximally placed tie of 4-0 suture. A sterile catheter introducer was used to guide the placement of the jugular catheter. A 4-0 ligature was placed at the distal end of the catheter to secure it in place. Sterile catheters were tested for patency by flushing with sterile saline. The catheter was tunneled subcutaneously to exit via an approximately 2.5 cm incision in the interscapular region. Wound clips and/or suture material were used to close the interscapular area. All surgeries and injections were standardized to avoid any additional variables. Sterile heparin–saline was injected using a standardized schedule to prevent clot formation within the catheter [36, 185].

Drug treatments

Alpha-Linolenic acid (LIN): LIN (Nu-Chek Prep Inc., Elysian, MN) was freshly prepared on the day of experimentation. Alpha-linolenic acid was dissolved in ethanol at a molar concentration and then diluted in NaCl 0.9% solution to reach a final concentration of 500 μM. The pH of the solution was adjusted to 7.0 for bolus intravenous injection. Vehicle (0.05% ethanol) was prepared in an identical fashion and served as the appropriate control for LIN-treated animals. The dose of LIN used in this study is 500 nmol/kg (LIN500) and was administered intravenously seven days, 3 days and 30 min prior to soman or 30 min, 3 days and 7 days after soman.

Soman: Male Sprague-Dawley rats (275-300 g) receiving nerve agent were injected with the oxime HI-6 (125 mg/kg, intraperitoneally [i.p.]), an acetylcholinesterase reactivator that restores activity in molecules that have not undergone aging [186], followed by soman (180 μg/kg, subcutaneously [s.c], 1.6xLD_{50}) 30 min later. This dose of soman was chosen because it reproducibly elicits seizures in 100% of the animals tested [36]. Seizures occur within 4–8 min after soman injection. Rats were monitored for behavioral signs of seizures and peripheral effects. Atropine methyl nitrate, an inhibitor of peripheral muscarinic receptors (2 mg/kg, intramuscuarlly [i.m.]), was administered 1 min after soman. Rats were allowed to seize for 40 min and then treated with the anticonvulsant diazepam (10 mg/kg, i.m.) to stop/attenuate the *status epilepticus*. The minimal duration of seizure activity necessary for irreversible damage is about 20 min, the damage process accelerates greatly after this minimal time has elapsed [187]. Atropine methyl nitrate and HI-6 do not readily cross the blood-brain barrier, but these agents effectively block OP-induced effects in the periphery [188]. The three drugs, HI-6, atropine methyl nitrate and diazepam, are employed to increase animal survival. After soman exposure, one group of rats was euthanized on day 10 to quantify neuronal degeneration. Another group were allowed to recover for two weeks, subjected to behavioral tests and euthanized on day 21 after soman exposure to quantify neuronal degeneration. In all cases, neuronal degeneration was quantified in the basolateral and centromedial amygdala, hippocampus, cingulate cortex and piriform cortex.

Behavior Measures: All behavior tests commenced on day 15 after soman/saline exposure.

Open field test: This test was measured using the Accuscan Superflex Sensor Version 2.2 infrared photocell system in the Accuscan Instruments Standard Animal Cage (measuring 40 x 40 x 30 cm; Accuscan Instruments Incorporated, Columbus, OH) located in a dedicated room designed to minimize acoustic interruptions. When testing, the room was illuminated with two infrared lights containing 830 nm filters that emit light that is not visible to rats. The test cage was constructed of Plexiglas with a ventilated, removable plexiglas lid that prevented the animal's escape during the trial but allowed adequate airflow. The animal's locomotion was captured by three, paired 16-photocell Superflex sensors, which transmit the location data to the Accuscan Superflex Node located on the upper-rear of the chamber. The Superflex Node transmits the open field data to the computer through a universal serial bus connection (USB). The data from the sixteen chambers was processed and aggregated by Accuscan Fusion Software (Version 3.4) on a computer located within the test room. On the first day (training day) animals were acclimated to the test cage for 1h. The next day (test day), the open field activity of each rat was measured for 1h. The total distance (cm) travelled was taken to provide a measure of general locomotor activity. The open field test equipment begins recording data immediately following the rat's entry into the chamber. Following the completion of the test, the rat was returned to its home cage and the test chamber is cleaned. The total distance measured in centimeters was recorded for all groups of animals and the results for each group of animals are plotted as total distance in cm ± SEM.

Forced swim test: The rat was placed in an inescapable glass cylinder (45 cm high, 20 cm diameter) filled with 30 cm of fresh water (28-32^{o}C) and a camera recorded its activity.

When testing, the room was illuminated with two infrared lights containing 830 nm filters not visible to rats. After an initial period of struggling, swimming and climbing, the animal eventually displays floating or an immobile posture. The time and the number of immobile episodes were recorded by computer software (any-maze, http://www.anymaze.com/). The forced swim test procedure occurred over two days and required two exposure times to water: Fifteen minutes on the first day (training day) and five minutes on the second day (test day). The proportion of immobility to mobility on the test day was considered a measure of the extent of learned helplessness in the animals exposed to the forced swim test. After each trial, the rats were dried before returning to their respective home cages. The time spent immobile in seconds was recorded for each animal/group, analyzed and plotted as the average immobility time in seconds ± SEM.

Rotarod test: Motor coordination, balance, fatigue resistance and motor learning were assessed using the rotarod [189-191]. Each rotarod treadmill consisted of a motor-driven drum with either constant speed or an accelerating speed mode of operation. The drum (7.0 cm diameter) allows each animal to maintain a suitable grip. It is divided into four test zones (8.9 cm wide) so that up to four animals may be tested at the same time. The device consists of a smooth hard plastic cylinder with concentric circular plastic dividers (39.7 cm diameter) attached to prevent the rat from climbing off the cylinder laterally. The device (Med Associates, Inc, St. Albans, VT) was set to accelerate to 33 rpm over the span of 3 minutes. Rats were placed on the device facing the wall with the rod rotating in the direction opposite from the animal so that the rat has to use its paws to pace forward to avoid falling off the rod. When a rat fell off the rotating drum (height of

26.7 cm), it breaks a photobeam, stopping the timer associated with that chamber. Each rat had three trials on the rotarod, separated by 5 min, and the average of the three attempts was recorded. After each trial, the animals are returned to their respective home cages and the apparatus cleaned. The amount of time before a rat fell off the wheel (i.e., latency), with a maximum of 3 min, was recorded, analyzed and plotted as average time the rats remain on the rotarod in seconds ± SEM.

Passive avoidance (PA) task: All animals were tested on a shuttlebox step-through passive avoidance task. The test apparatus (Gemini model, San Diego Instruments, San Diego, CA) consisted of two chambers (21 cm x 25 cm x 17 cm). A 50 watt light bulb was present in one of the compartments. The training and testing operations were controlled by computer software ("PA", San Diego Instruments, San Diego, CA). During the training period, rats were placed in a chamber. After 60 seconds, the light in that chamber was turned on and the door to the second dark chamber was opened. Upon crossing into the dark chamber, the door was closed and a single 0.8 mA shock was delivered through a grid floor to the animal for a period of one second. On the test day, the rats were again placed in the chamber and after 60 seconds, the light in that chamber was again turned on and the door to the second chamber was opened. The amount of time it takes for the rat to cross from the light to the dark chamber is referred to as the retention latency and is recorded by the computer. Each animal is removed after 300 seconds. Testing was performed 24 hours and 5 days after the training period. The procedure for the testing period was identical, except that the rats are not shocked upon crossing to the dark side of the chamber. A maximum retention latency of 300 seconds

was given to rats that did not enter the dark compartment before that time. After each trial, the animals were returned to their respective home cages and the apparatus is cleaned. The retention latency of a single trial during each testing period was recorded for all animals in each group and the results were analyzed and plotted as average retention latency in seconds ± SEM.

Histological procedures:

On day 10 or 21 after soman or saline injection, all groups of animals were deeply anesthetized with pentobarbital (50 mg/kg, i.p.) and transcardially perfused with phosphate-buffered saline (PBS, 100 ml) followed by 4% paraformaldehyde (250 ml) in PBS. The brains were removed and post-fixed overnight at 4°C, transferred to a solution of 30% sucrose in PBS for 72 h and frozen on dry ice prior to storage at −80°C until sectioning. Coronal brain sections (40 μm) were prepared on a cryostat (Leica Microsystems, Bannockburn, IL), and one section in every 1-in-15 series was collected in 0.1 M neutral phosphate buffer, mounted on slides (6 sections per slide) (Superfrost Plus; Daigger, Vernon Hills, IL) and air dried.

Fluorojade C staining: The staining was performed in accordance with the manufacturer's instructions (Histo-Chem, Jefferson, AK) and as described previously [36]. Briefly, slides containing formalin-fixed brain sections were transferred to a solution of 0.06% potassium permanganate for 10 min on a shaker table to ensure consistent background suppression between sections. The slides were then rinsed in deionized water for 2 min and the Fluorojade C Staining (FJC) solution was added for 10 min in the dark. The

slides were rinsed for 1 min in each of three deionized water washes and rapidly placed on a slide warmer, set at approximately 50°C, until they were fully dry (about 20 min). The dry slides were cleared by immersion in xylene for at least 1 min before coverslipping with DPX (Sigma–Aldrich, St. Louis, MO), a non-aqueous non-fluorescent plastic mounting media.

Quantification of neuronal degeneration:

Three representative fields from each brain region per animal were randomly captured using a 300 color CCD camera (AxioCam, Carl Zeiss International, Thornwood, NJ). The camera was attached to an Axiovert 200 microscope equipped with epi-fluorescence using an excitation wavelength of 490 and emission wavelength of 525 (Carl Zeiss International, Thornwood, NJ). Neuronal degeneration was quantified by counting the number of FJC-positive neurons in the cingulate cortex, basolateral and centromedial amygdala, CA1 and CA3 subfields of the hippocampus and piriform cortex. The coordinates of brain structures are based on Paxinos and Watson (2005). The percent of neuronal degeneration was determined according to the following formula and as previously described [36, 185].

$$\frac{\text{average \# of FJC-positive neurons in LIN-treated animals}}{\text{average \# of FJC-positive neurons in soman-treated animals}} \times 100$$

Percent neuronal degeneration in soman-treated animals was arbitrarily set at 100% [36, 185].

Statistical analysis

All values were expressed as the mean ± SEM. Sample sizes (*n*) refer to the number of rats included in each analysis. The statistical evaluation of the results was carried out using two-way repeated measures analysis of variance (ANOVA) for changes in body weight, one-way ANOVA for behavioral tests, the t test for neuronal degeneration and Fisher's exact test for overall animal survival. Following significant ANOVA, *post hoc* comparisons were performed using the Bonferroni and Tukey test. The level of significance for all tests is $p < 0.05$. All tests were performed using the Graphpad prism 6.0 software.

RESULTS

The two experimental designs used in this study comprise of a pre-treatment and post-treatment LIN500 schedule (Figure 1) to evaluate the effects of LIN500 treatment on behavioral outcomes, neuronal degeneration and animal survival. The average changes in body weight observed in rats after soman exposure pretreated with LIN500 (Figure 2A) or post-treated with LIN500 (Figure 2B) demonstrate a statistically significant weight loss in the soman-exposed animals compared to saline/vehicle and saline/LIN500 groups of animals during the first three to six days ($p<0.05$) after soman exposure. The dosing schedule of LIN500 did not itself affect the weight loss and there were no statistically significant differences among the saline-treated groups of animals (Figure 2). By day 15, animals demonstrated similar body weights and were not statistically significantly different between groups. Rats with excessive weight loss (more than 25%) were not used in this study (*n*=2 from soman/veh group).

Behavioral studies

All rats were first tested in the open field test on day 15 after soman exposure to determine their locomotor activity, which is a preliminary measure for motor impairment or weakness in rodents [46]. The average distance traveled (cm) is shown for groups of animals pretreated (30 min, 3 days, 7 days prior to soman [Figure 3A]) or post-treated (30 min, 3 days, 7 days after soman [Figure 3B]) with intravenous LIN500. There were no significant differences in locomotor activity among groups of animals exposed to soman that were either pretreated or post-treated with LIN500 compared with saline/vehicle or saline/LIN500 groups of animals. These results are comparable to previous work (Buccafusco et al., 1990).

Effect of LIN500 treatment on soman-induced behavioral and motoric alterations

Depressive behavior was tested in the modified version of Porsolt's forced swim test, a widely used test to evaluate antidepressant efficacy [60, 61]. The time spent immobile has been identified as correlating with depressive-like activity and can be reduced by antidepressants drugs [60]. Soman exposure exerts a profound effect on the forced swim test immobility time. Rats exposed to soman spent significantly more time immobile [pre-treatment $F(3, 30) = 8.826$ $p < 0.05$) and post treatment ($F(3, 32) = 6.784$, $p < 0.05$)] compared to saline/vehicle and saline/LIN500 groups of animals (Figure 4A and B). Administration with three injections of intravenous LIN500 before soman exposure (LIN500/soman; pre-treatment) showed no statistically significant difference ($p > 0.05$) in the forced swim test compared to the vehicle/soman exposure groups of animals (Figure 4A). In sharp contrast, administration of LIN500 given via intravenous injection 30 min, 3 days and 7 days after soman exposure (soman/LIN500) significantly

reversed the soman-induced increase in immobility time by 3.8-fold in the forced swim test ($p < 0.05$) [Figure 4B]. There were no statistically significant differences between saline/vehicle, saline/LIN500, and soman/LIN500-treated groups of animals (Figure 4A and B).

The rotarod test has been used to evaluate motor learning in various models of brain injury [19, 192, 193]. Soman-exposed rats administered with intravenous vehicle perform poorly on the rotarod test [pre-treatment $F(3, 92) = 17.63$ $p < 0.05$) and post treatment ($F(3, 106) = 10.21$, $p < 0.05$)] compared to saline/vehicle and saline/LIN500 groups of animals (Figure 4C and D). Administration with three injections of intravenous LIN500 before soman exposure (pre-treatment) showed no statistically significant difference in the performance on the rotarod test compared to the groups of animals exposed to soman/vehicle (Figure 4C). However, administration of three doses of intravenous LIN500 after soman improves performance, with a significant increase of 2.9-fold on time spent on a rotating rod ($p<0.05$). No statistically significant differences were found among saline/vehicle and saline/LIN500 groups of animals (Figure 4C and D).

Effects of LIN500 treatment on soman-induced impairments in the passive avoidance test

The passive avoidance test is an index of learning and general cognition performed in a two compartment (light and dark) shuttle step-through box [194, 195]. Because rodents prefer darkness, the animal has to suppress this tendency through pairing of an aversive stimulus (such as a foot-shock) within the desired compartment. Animals that do not remember the aversive stimulus will cross over earlier than animals that do

remember and will have a lower retention latency. Analyzed data from the passive avoidance test 24h after training from the soman groups of animals that received intravenous vehicle or LIN500 showed no statistically significant difference in retention latency compared to the saline groups of animals (Figure 5). However, there is a significant decrease in retention latency in the soman group of animals that received intravenous vehicle either prior to or after soman when tested five days after training [pre-treatment $F(3, 11) = 6.519$ $p < 0.05$) and post treatment ($F(3, 20) = 8.128$, $p < 0.05$)] compared to saline groups of animals (Figure 5A and B). Administration of LIN500 by intravenous injection 30 min, 3 days and 7 days prior to soman (pre-treatment) showed no statistically significant difference compared to the soman group of animals that received intravenous vehicle (Figure 5A). Also there were no statistically significant differences between the LIN500/soman-treated animals compared to the veh/saline and LIN500/saline groups of animals. In contrast, administration of LIN500 intravenously 30 min, 3 days and 7 days after soman exposure significantly reversed the soman-induced decrease in retention latency five days after training ($F(3, 20) = 8.128$, $p<0.05$). There were no statistically significant differences among saline/vehicle and saline/LIN500 groups of animals (Figure 5A and B).

Effect of LIN500 treatment on soman-induced neuronal degeneration.

We have previously demonstrated that administration of a single dose of LIN (500 nmol/kg, i.v.) either prior to or after soman exposure significantly protected against the neuropathological damage induced by soman 24h later in the piriform cortex, amygdala, hippocampus and prefrontal cortex, using the Fluorojade C staining method (Pan et al.,

2012). In this study, neuronal degeneration was quantified twenty-one days after soman exposure, in the amygdala, hippocampus, piriform cortex and cingulate cortex in animals administered three doses of LIN by intravenous (i.v.) injection either prior to (pre-treatment) or after (post-treatment) soman exposure. In the experimental groups of animals administered vehicle followed by exposure to soman, there was a significant increase ($p<0.05$) in the percent neuronal degeneration 21 days after soman in the four brain regions most vulnerable to nerve-agent exposure compared to saline/vehicle and saline/LIN500 groups of animals (Figures 6-8). Administration of three doses of intravenous LIN500 prior to soman exposure (pre-treatment group) and euthanized at day 21 after soman showed no statistically significant difference compared to the soman group of animals that received intravenous vehicle (Figure 6).

Because administration of three intravenous injections of LIN500 prior to soman did not reduce the ongoing neuronal degeneration at day 21, we first determined whether the three doses of LIN post-treatment reduced neuronal degeneration ten days after exposure to soman. Administration of LIN500 injected intravenously at 30 min, 3 days and 7 days after soman resulted in a significant reduction in the number of FJC-positive neurons in the cingulate cortex (32%, Figure 7A), hippocampus (72%, Figure 7B), amygdala (43%, Figure 7C) and piriform cortex (46%, Figure 7D) on day 10. In order to determine whether the reduction in neuronal degeneration continued beyond day 10, neuronal degeneration was quantified on day 21 after soman exposure. A significant reduction in neuronal degeneration from animals that received intravenously LIN500 30 min, 3 days and 7 days after soman is observed in the cingulate cortex (42%, Figure 7E), hippocampus (62%, Figure 7F), amygdala (29%, Figure 7G) and piriform cortex (56%,

Figure 7H) on day 21 after soman compared to soman followed by three injections of intravenous vehicle.

Representative photomicrographs of the cingulate cortex, hippocampus, amygdala and piriform cortex on day 21 are illustrated from the post-treatment groups of animals. Soman exposure results in a marked increase in the number of fluorescein-positive neurons (degenerating neurons [arrowheads]) in the cingulate cortex, hippocampus, amygdala and piriform cortex. In contrast, post-treatment with three doses of LIN500 following soman exposure markedly reduces the number of degenerating neurons (Figure 8 A-D).

Administration of three doses of LIN500 after soman increases animal survival rate.

Overall animal survival was analyzed over the twenty-one days of the experiment in groups of animals pretreated or post-treated with three doses of LIN500. Administration with three injections of intravenous LIN500 before soman exposure (pre-treatment) showed no statistically significant difference (Figure 9A) in animal survival compared to the groups of animals exposed to soman. However, administration of three doses of intravenous LIN500 after soman exposure significantly improves overall survival of rats ($p<0.05$) compared with groups of animals exposed to soman/vehicle (Figure 9B). There were no statistically significant differences among saline/vehicle and saline/LIN500 groups of animals (Figure 9A and B).

DISCUSSION

The results of the current study provide a number of novel insights into the long-term effects of LIN treatment on soman-induced behavioral alterations and associated neuronal degeneration. In this study, we used three widely employed behavior measures and compared groups of animals that were pretreated or post-treated with LIN500 or vehicle to those exposed to soman. The first test, the Porsolt forced swim test, is a model of behavioral despair and a widely accepted test that predicts antidepressant efficacy [61]. For the first time, we demonstrate that the nerve agent soman significantly increases immobility time in the forced swim test. Other organophosphorous compounds that inhibit AChE activity, such as malathion, have also been shown to increase immobility time in the forced swim test [62]. The increase in immobility time in the forced swim test after soman exposure cannot be attributed to changes in locomotor performance since no significant differences were apparent in soman-exposed rats in the open-field test compared to respective controls. These results are comparable to clinical observations of deployed veterans exposed to nerve agents [54, 56] and victims of the terrorism attack in Japan [55] showing neuropsychiatric disorders involving emotional behavior. Increased despair in the forced swim test after soman exposure is likely to be a consequence of neuronal loss in limbic brain structures, as evidence indicates that mood disorders are characterized by enhanced neurodegeneration [196-198]. Moreover, structural brain changes from follow-up patients from the Tokyo sarin attack who developed PTSD revealed a smaller amygdala volume and demonstrated dysfunction in the amygdala and anterior cingulate cortex [57, 174]. It has been also been suggested that a reduction in hippocampal neurogenesis causes depression in adults [71] but this hypothesis has been recently questioned [88]. Previous findings demonstrated that administration of three

doses of LIN500 at 30 min, 3 days and 7 days exerts an anti-depressant-like activity and an increase in neuroplastic effects [110]. In the present study, we hypothesized that three doses of LIN500 administered over the identical time frame would produce similar effects in a brain damaged by soman. Interestingly, post-treatment but not pretreatment with three doses of LIN500 after soman resulted in beneficial effects on reversing the depressive-like behavior in soman-treated animals. These results are in line with the anti-depressant effects of omega-3-enriched diets [199-201]. Our results also demonstrate that administration of three doses of LIN500 after but not before soman challenge profoundly reduce neuronal degeneration, suggesting that there may be an association between the reduced neuronal degeneration and the improvement in functional outcome. In addition, this treatment offers a relatively short duration but a long-lasting anti-depressant-like activity, lasting ten days after the third dose of LIN.

In the current investigation, performance on the rotarod task was used to assess motor function following soman exposure. In this study, soman-treated animals spent less time on the rotarod compared with controls – an effect opposite to that previously found in Sprague-Dawley rats exposed to soman (1.0 and 1.2 LD_{50}) [52]. It is possible that the difference in the dose of soman injected into the animals accounts for this differential effect. A biphasic change in motor activity was observed during 24 hours and 21 days after exposure to sublethal doses of soman [202] and other nerve agents like sarin have been reported to impair motor performance in rodents [203, 204]. As observed in the forced swim test, administration of LIN500 after, but not prior to soman increased the amount of time that the rats spent on the rotarod and were comparable to their control counterparts. Thus, administration of three doses of LIN500 after soman exposure

markedly reduced motoric impairment on the rotarod and this effect was associated with reduced neuronal degeneration twenty-one days after soman exposure.

The passive avoidance task was chosen to test learning and memory because it relies on the hippocampus and amygdala regions to be successfully performed [68, 69]; the amygdala and hippocampus are two brain regions that show profound damage in soman-exposed animals [36]. Retention latency analyzed 24h after training showed no impairment in soman/vehicle, LIN500/soman, soman/LIN500 or saline controls indicating that memory consolidation occurred in the brain. However, a deficit in memory retention was evidenced by reduced retention latency in the soman/vehicle group of animals when tested five days after training in the soman group of animals compared to saline/vehicle and saline/LIN500 groups of animals. Other Pavlovian tasks involving learning and memory in the hippocampus and amygdala such as fear conditioning are also impaired in rats exposed to soman and were associated with neuronal loss and degeneration in the amygdala [52, 205]. Our study used a soman dose of $1.6xLD_{50}$ which is similar with that used in previous reports [52, 205]. Moreover, neuronal loss in the hippocampus correlates with significant impairments in the passive avoidance task after kainate-induced SE in rats [206]. The present work is consistent with these findings, which attribute the soman-induced deficits in memory retention to neuronal degeneration found in the hippocampus and amygdala.

The effects of soman on the passive avoidance task were counteracted by LIN500 treatment 30 min, 3 days and 7 days after soman exposure suggesting that newly acquired memory was retained by this group of animals; administration of three injections of LIN500 after soman exposure also markedly reduces neuronal degeneration in the

amygdala and hippocampus. Pretreatment with LIN500, however, did not improve memory retention when animals were tested five days after training and did not reduce neuronal degeneration in the amygdala and hippocampus. Dietary fatty acids supplementation, especially omega-3 fatty acids, has proven beneficial to enhance learning and memory and prevent memory deficits under various experimental conditions [207-210]. In particular, LIN has been shown to stimulate brain-derived neurotrophic factor (BDNF) induction and increase neurogenesis in the hippocampus [110, 211] both of which have been proposed to play important roles in learning and memory. BDNF is a neurotrophic factor essential for maintenance of neuronal integrity and neuronal plasticity that affects behavior [212]. Furthermore, BDNF signaling enhances hippocampal neurogenesis [127, 213, 214]. Newborn neuronal cells from the subgranular zone of hippocampus migrate and differentiate extending new axonal processes and establish synaptic connections with neighboring CA3 cells which may enhance hippocampal learning and memory [76, 215]. This hypothesis is further supported by the finding that BDNF and neurogenesis are involved in improved performance of the passive avoidance task [91]. Whether BDNF and downstream signaling mechanisms play a role in the demonstrated neuroprotective and neurorestorative efficacy of LIN500 against soman-induced cognitive and behavior impairments remains to be determined.

The present study also demonstrated that administration of three doses of LIN500 injected intravenously after soman exposure significantly reduced neuronal degeneration 24 hours [36] 10 and 21 days after soman exposure compared with soman-treated groups of animals whereas neuronal degeneration remained unaffected in groups of animals pretreated with LIN500 followed by soman.

The soman-induced neuropathology observed in this study is consistent with previous studies demonstrating neuropathological changes in several brain regions in rodent brain up to three months following soman exposure [20, 205, 216]. The neuronal damage that continues for days, weeks and months after OP exposure has also been shown to increase the behavior deficit [217, 218] suggesting that ongoing neurodegeneration plays a key role in the pathophysiological consequences following soman exposure.

Neuronal degeneration in the soman group of animals was markedly evident despite life-saving treatment with atropine, HI-6 and diazepam as previously described [16, 171]. These results confirm previous work demonstrating that initial drug therapies are not sufficient to prevent the brain damage caused by prolonged seizures induced by nerve-agents [219-221]. Our results demonstrate continued neuronal cell death up to at least three weeks after soman exposure, suggesting that processes other than the initial insult are responsible for the delayed damage as previously described [20]. Surprisingly, administration of three doses of LIN500 prior to soman exposure did not significantly reduce neuronal cell death twenty-one days after soman exposure. These results show that the timing of alpha-linolenic acid administration is a crucial factor and demonstrate that the alpha-linolenic acid needs to be administered after the acute brain injury in order to exert its neuroprotective and neurorestorative efficacy.

The accumulation of synaptic acetylcholine leading to status epilepticus and the increase of glutamate resulting in hypoxic-ischemic injury can be partially responsible for the neuronal death found in the brain regions known to be vulnerable to soman [16, 34, 165, 222]. Overactivation of NMDA receptors and the massive influx of calcium through

its voltage-gated calcium channel leads to the activation of necrotic pathways [17, 31, 32, 37, 223]. We refer to this initial cascade of events as the primary insult leading to neuronal cell death. One possible mechanism of LIN-induced protection against the primary insult is via the activation of background rectifying potassium channels – TREK-1, TREK-2 and TRAAK [109]. Activation of background potassium channels would be expected to hyperpolarize neuronal membranes thereby decreasing the release of the excitatory neurotransmitter glutamate that would in turn reduce the overactivation of NMDA ionotropic glutamate receptors leading to neuronal cell death. The results demonstrating that LIN does not protect the hippocampus against kainic acid-induced neuronal cell death in TREK 1 knockout mice, confirm the potent and robust effect of these rectifying background channels in reducing NMDA receptor-mediated excitotoxicity [224]. Other possible mechanisms of LIN-induced neuroprotection include the inhibition of voltage sensitive sodium [225] and calcium channels [226], which may also contribute to reduce glutamate-mediated excitotoxicity.

We also examined whether LIN treatment exerts a therapeutic effect on the survival of soman-exposed rats. Soman-induced seizures rapidly progress leading to convulsions, coma, and death [16]. To increase animal survival, animal models of OP toxicity receive pharmacological treatment with oximes, atropine and diazepam, to attenuate seizures and reduce peripheral signs of OP toxicity although recurrent seizures may occur [16, 33]. However, in rats and in humans, these drug treatments are not effective in preventing the brain damage and eventual death. In this study, we found that LIN administration after but not prior to soman significantly improved animal survival by

30%, 3 weeks after soman exposure. This effect may be explained by the activation of multiple survival pathways mediated by the pleiotropic effects of LIN [227].

CONCLUSION

Our results demonstrate that the timing of administration of LIN relative to soman exposure is critical for LIN to exert long-term neuroprotective efficacy. An important and significant improvement was found in the behavior tests using three doses of LIN500 administered after soman exposure. The mechanism(s) of LIN in ameliorating the cognitive deficits remains unclear, but a significant reduction in neurodegeneration appears to be an important target to reduce cognitive deficits. Evidence from our laboratory has demonstrated an increase in BDNF and neurogenesis in the hippocampus of a normal brain after LIN treatment using the identical dosing schedule which may underlie the long-term benefit of this compound. OPs and other brain injuries that lead to depression and cognitive deficits affect the lives of millions of people world-wide and are often incapacitating, leading to significant cognitive and neuropsychiatric dysfunction. It would, therefore, be of interest to define which targets of LIN contribute most to its efficacy in promoting long-lasting neuronal survival and improvement in the behavior tests. Identifying these mediators would open new avenues of drug discovery that would positively modulate these targets to reduce the cognitive and neuropsychiatric deficits and improve quality of life.

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FIGURES LEGEND

Figure 1. Experimental design diagram. The pretreatment paradigm consisted of administering alpha-linolenic acid (LIN500) via the intravenous route at seven days, 3 days and 30 min prior to soman while the post-treatment paradigm consisted of administering LIN500 via the intravenous route at 30 min, 3 days and 7 days after soman exposure.

Figure. 2. The effect of soman and alpha-linolenic acid on body weight of young adult Sprague-Dawley male rats. Body weight is plotted versus time after soman for all groups of animals consisting of pretreatment (A) or post-treatment (B) with three doses of alpha-linolenic acid (LIN500) or vehicle (veh), followed by soman or saline exposure. The rats were weighed daily after the soman or saline injection (day 0) throughout the duration of the experiment. The dotted line represents the day that the behavior tests commenced. Values are expressed as mean ± SEM. *$p < 0.05$ or **p< 0.01 soman/veh *vs* saline/veh and $^{\dagger}p < 0.05$ or ††p< 0.01 soman/LIN *vs* saline/veh group. (n = 8-10/group).

Figure 3. **The effect of soman in the presence or absence of alpha-linolenic acid on the general health of young adult Spague-Dawley male rats.** Locomotor activity measured as the total distance traveled in the open field test was performed on day 15 and 16 after soman or saline exposure for groups pretreated (A) or post-treated (B) with alpha-linolenic acid (LIN500) or vehicle (veh). Values are expressed as mean cm ± SEM. (n = 8-10/group).

Figure 4. Effect of alpha-linolenic acid on the Porsolt forced swim test and rotarod test. Alpha-linolenic acid (LIN500) or vehicle (veh) was administered intravenously 7 days, 3 days and 30 min prior to or 30 min, 3 days and 7 days after soman/saline exposure. Performance in the Porsolt forced swim test was measured as time spent being immobile in seconds (s) performed at day 17 and 18 after soman or saline exposure for groups pretreated (A) or post-treated (B) with LIN500 or veh. ***p<0.001 vs saline/veh or saline/LIN group, **p < 0.01 vs saline/veh or saline/LIN group. #p <0.05 vs soman/veh (n = 8-10/group). Time spent on the rotarod in seconds [s] (average of three trials) was performed at day 15 after soman or saline exposure for groups pretreated (C) or post-treated (D) with LIN500 or veh (n = 8-10/group). ***p<0.001 vs saline/veh or saline/LIN group. #p <0.05 vs soman/veh group. Values expressed are as mean ± SEM.

Figure 5. Effect of alpha-linolenic acid on the passive avoidance task. The passive avoidance task was performed at day 16, 17 and 21 after soman or saline exposure for groups pretreated or post-treated with alpha-linolenic acid (LIN500) or veh. There was no statistical difference among groups in retention latency measured in seconds (s) 24h after training for groups pretreated (A) (n = 5-7/group) or post-treated (B) (n = 10-11/group) with LIN500 or veh. Five days after training, the soman/veh group shows a significant deficit of retaining the memory linked to the learning experiment and this effect is prevented for groups post-treated with LIN500 (B) (n= 5-7/group) but not in the group of animals pretreated with LIN500 (A) (n=5-9/group). *p < 0.05 or **p<0.01 *vs* saline/veh or saline/LIN group. #p <0.05 *vs* soman/veh group. Values are expressed as mean ± SEM.

Figure 6. Effect of pretreatment with alpha-linolenic acid on neuronal degeneration twenty-one days after soman exposure. The bar graphs show percent neuronal degeneration in four brain regions known to be vulnerable to injury after soman exposure from rats euthanized on day 21 from groups of animals pretreated with either alpha-linolenic acid (LIN500) or vehicle 7 days, 3 days and 30 min prior to soman or saline (A-D). The number of FJC-positive neurons was arbitrarily set at 100% neurodegeneration in the soman/veh group. Data are plotted as the average percent of neuronal degeneration ± SEM (n = 5/group).

Figure 7. Quantification of neuronal degeneration from animals post-treated with alpha-linolenic acid on day 10 or 21 after soman exposure. The bar graphs show percent neuronal degeneration in four brain regions known to be vulnerable to injury after soman exposure followed by treatment with either alpha-linolenic acid (LIN500) 30 min, 3 days and 7 days or vehicle (veh) and euthanized on day 10 (A-D) or on day 21 (E-H). The number of FJC-positive neurons was arbitrarily set at 100% neuronal degeneration in soman/veh group. Data are plotted as the avg percent neuronal degeneration ± SEM. *p <0.05 or **p<0.01 vs with soman/veh group (n = 5/group).

Figure 8. Representative photomicrographs of Fluoro-jade-C-positive stained degenerating neurons acquired from brain regions vulnerable to soman; the protective effect of post-treatment with alpha-linolenic acid. The brain regions that were quantified for neuronal degeneration are indicated in the coronal sections. Representative photomicrographs at 10x magnification were acquired from the cingulate

cortex (A), hippocampus (B), centromedial and basolateral amygdala (C) and piriform cortex (D) from animals post-treated with three doses alpha-linolenic acid (LIN500) or vehicle (veh) administered intravenously 30 min, 3 days and 7 days after soman exposure and euthanized on day 21. Cx=cingulate cortex, Hipp=hippocampus, Amyg=amygdala, PirC=piriform cortex. Degenerating neurons are indicated by the arrowheads. The cingulate cortex, hippocampus, piriform cortex and amygdala are shown in the coronal maps of the rat brain [228].

Figure 9. Effect of alpha-linolenic acid on animal survival after soman exposure. Overall survival for groups of animals exposed to soman or saline and either pre-treated (A) or post-treated (B) with alpha-linolenic acid (LIN500) or vehicle (veh). ***$p < 0.001$ *vs* saline/veh or saline/LIN group. $^{\#}p < 0.05$ *vs* soman/veh group. In the pre-treatment groups: n=9 for the saline groups and n=23-31 for the soman groups). In the post-treatment groups: n=10 for the saline groups and n=16-38 for the soman groups).

Figure 1. Experimental design diagram.

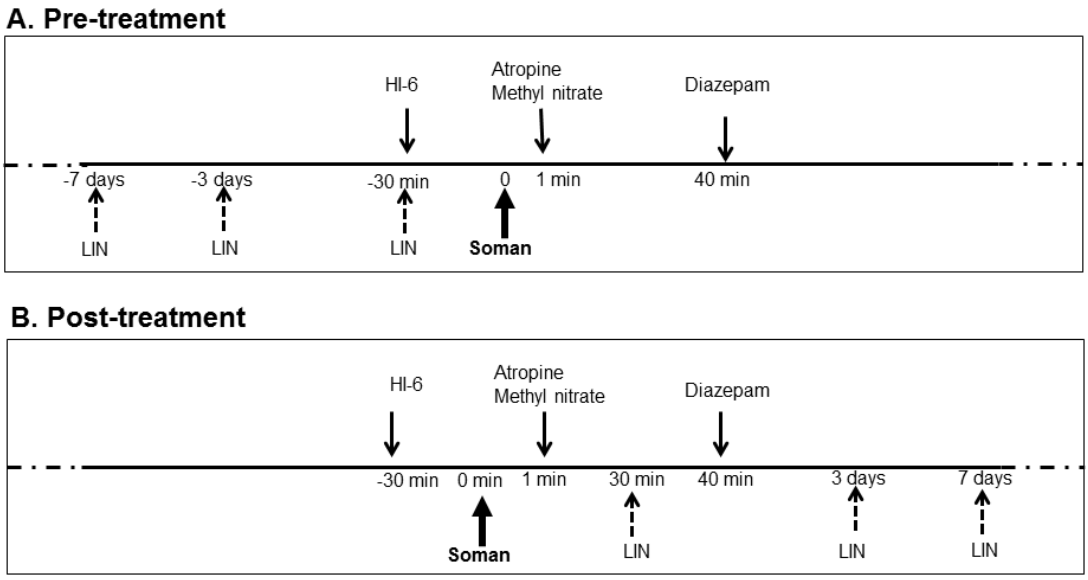
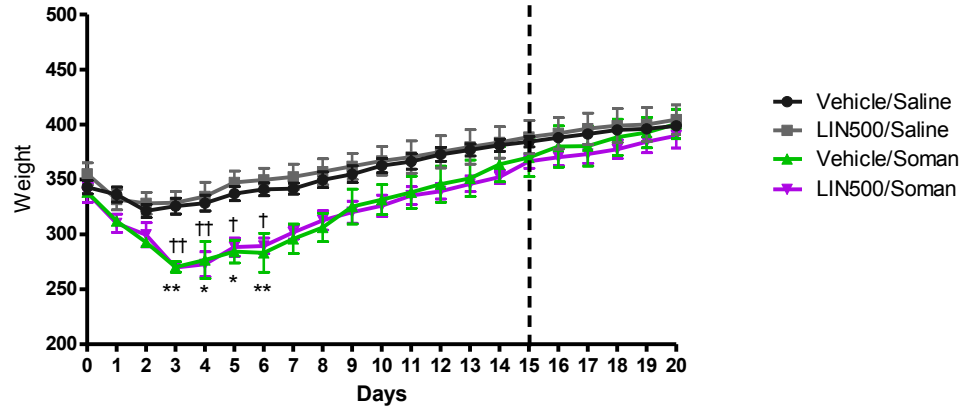


Figure 2. The effect of soman and alpha-linolenic acid on body weight of young adult Sprague-Dawley male rats.

A



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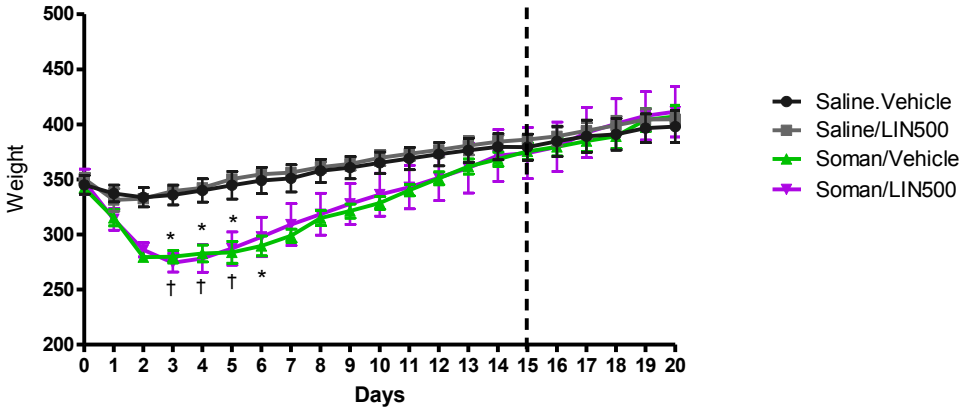


Figure 3. The effect of soman in the presence or absence of alpha-linolenic acid on the general health of young adult Spague-Dawley male rats.

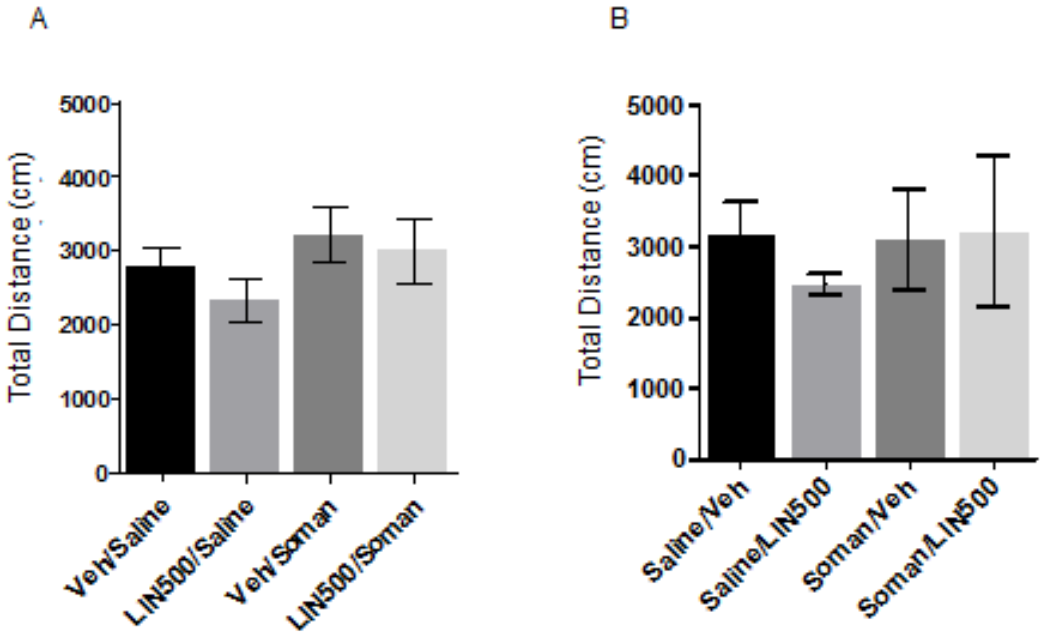


Figure 4. Effect of alpha-linolenic acid on the Porsolt forced swim test and rotarod.

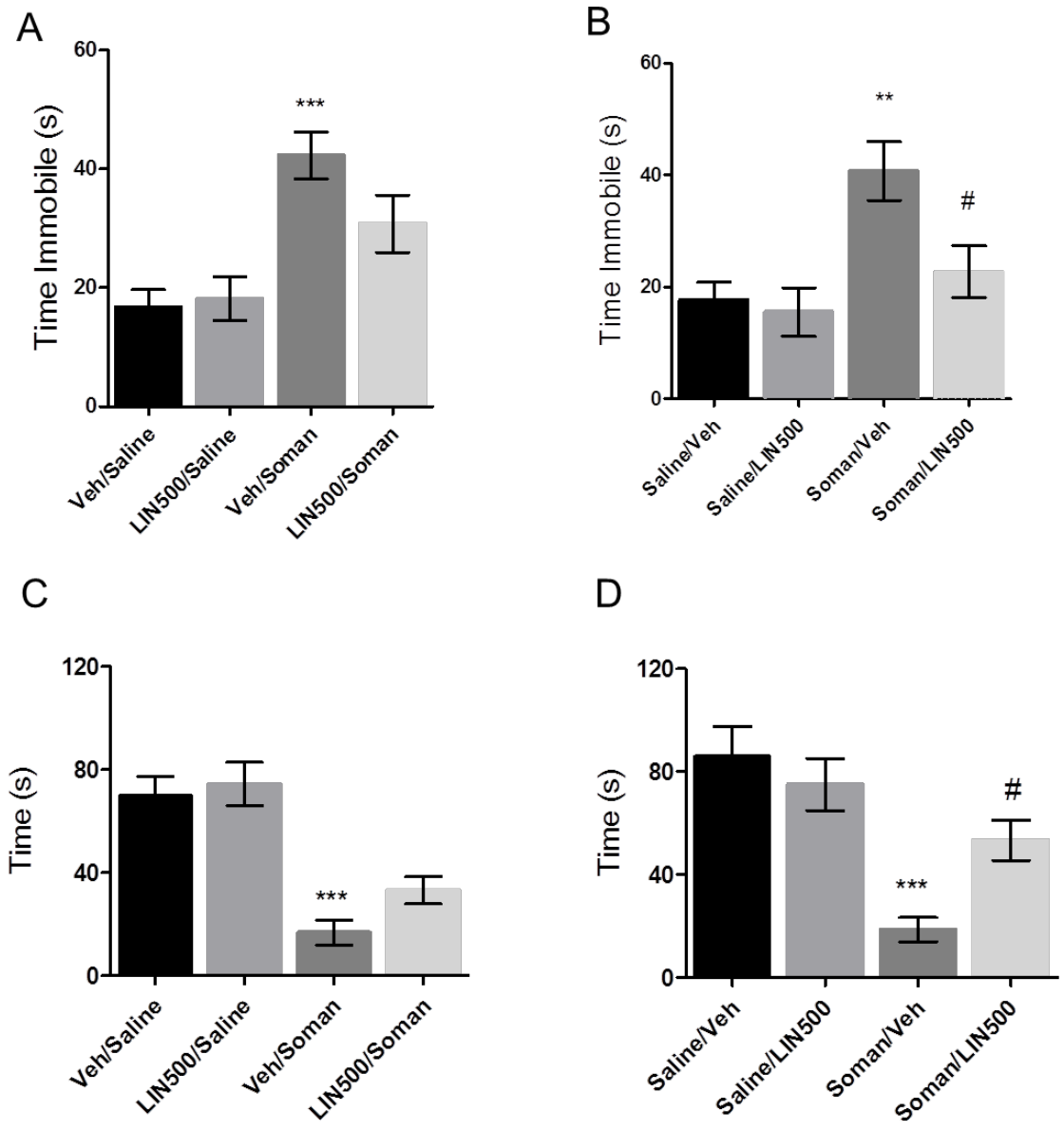


Figure 5. Effect of alpha-linolenic acid on the passive avoidance task.

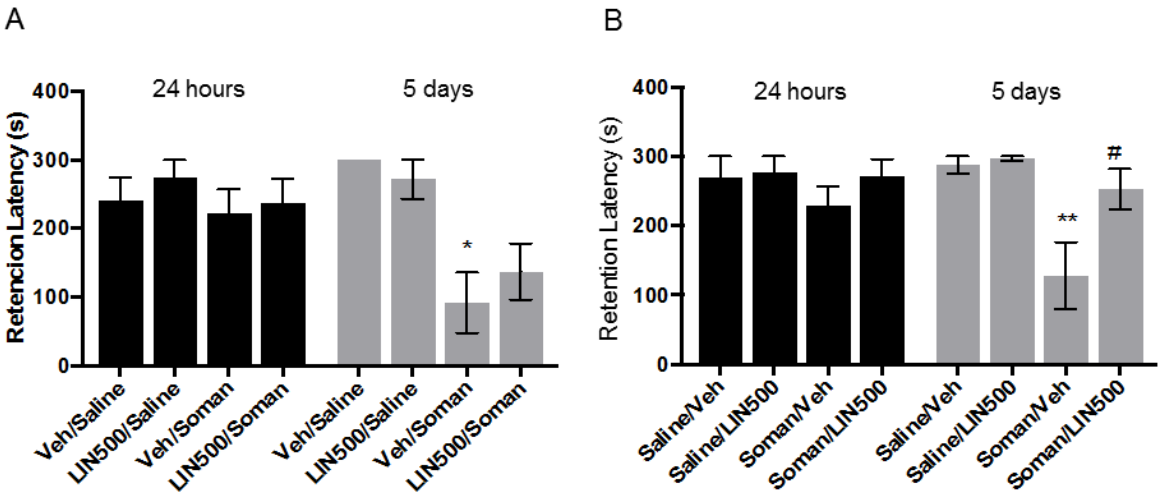


Figure 6. Effect of pretreatment with alpha-linolenic acid on neuronal degeneration twenty-one days after soman exposure.

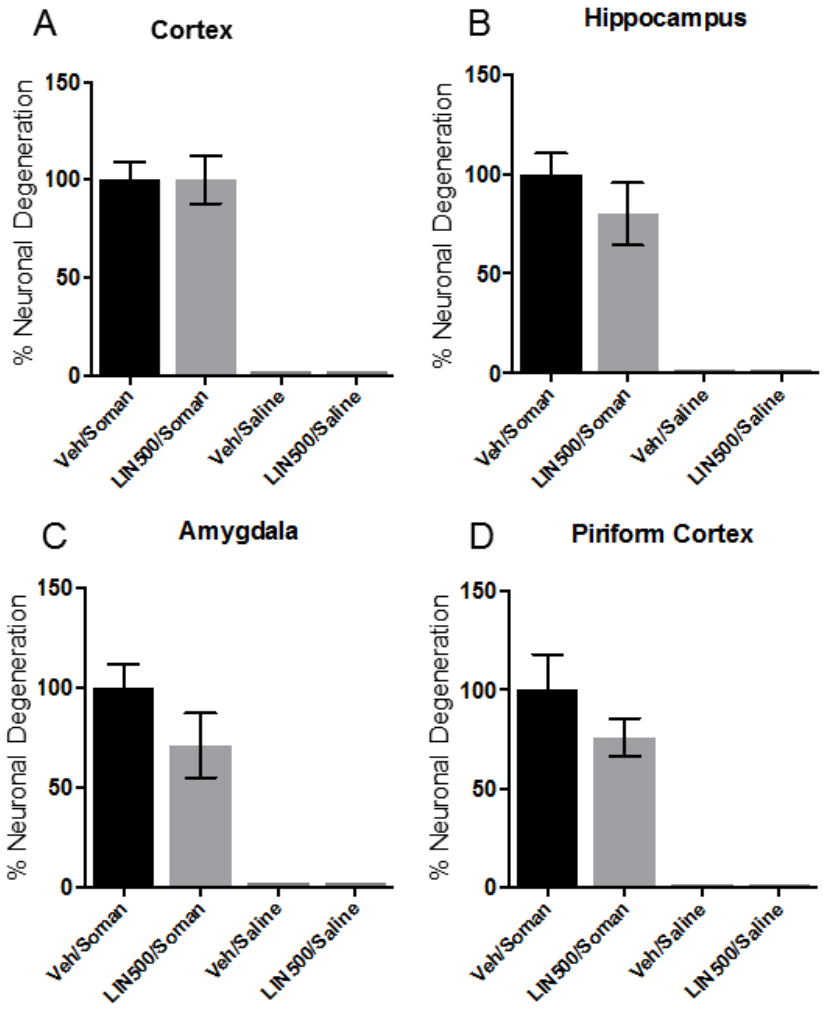


Figure 7. Quantification of neuronal degeneration from animals post-treated alpha-linolenic acid on day 10 or 21 after soman exposure.

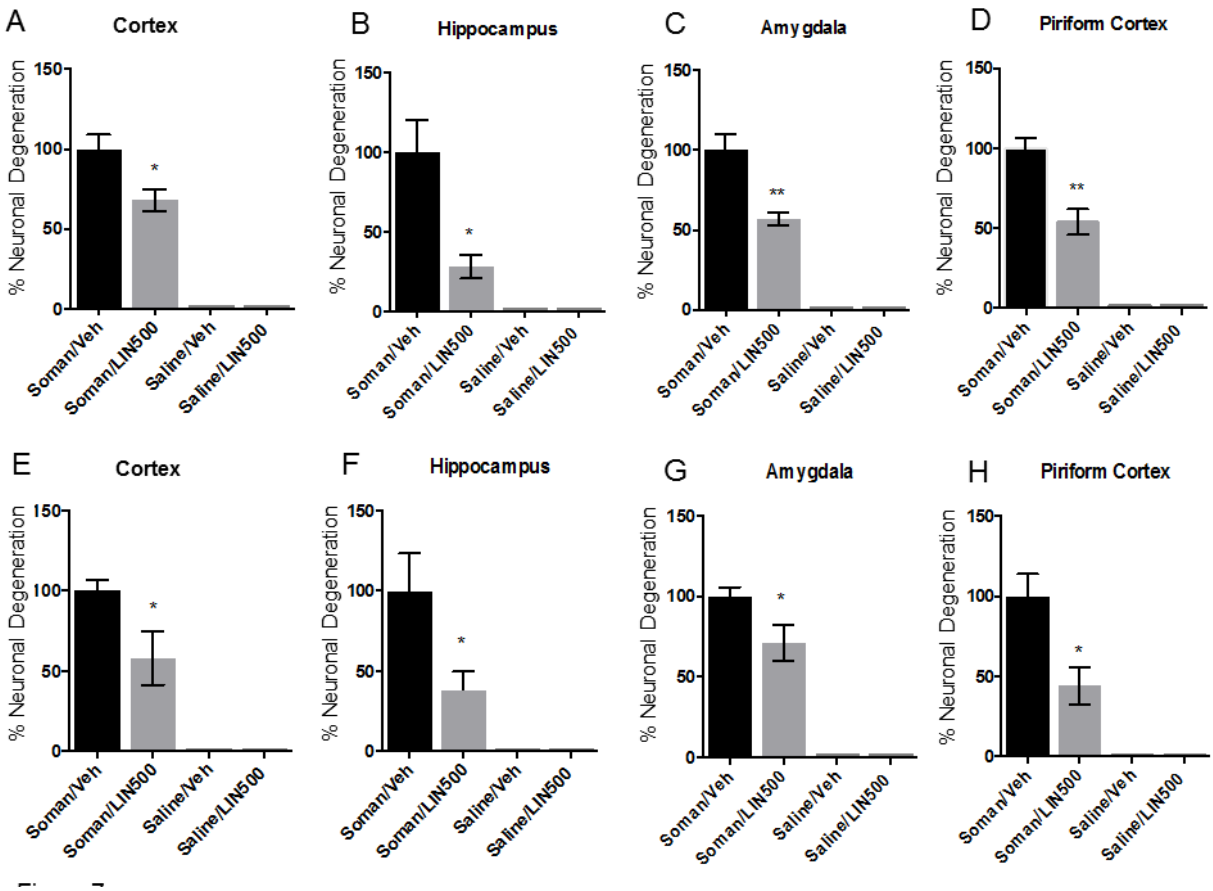


Figure 8. Representative photomicrographs of Fluoro-jade-C-positive stained degenerating neurons acquired from brain regions vulnerable to soman; the protective effect of post-treatment with alpha-linolenic acid.

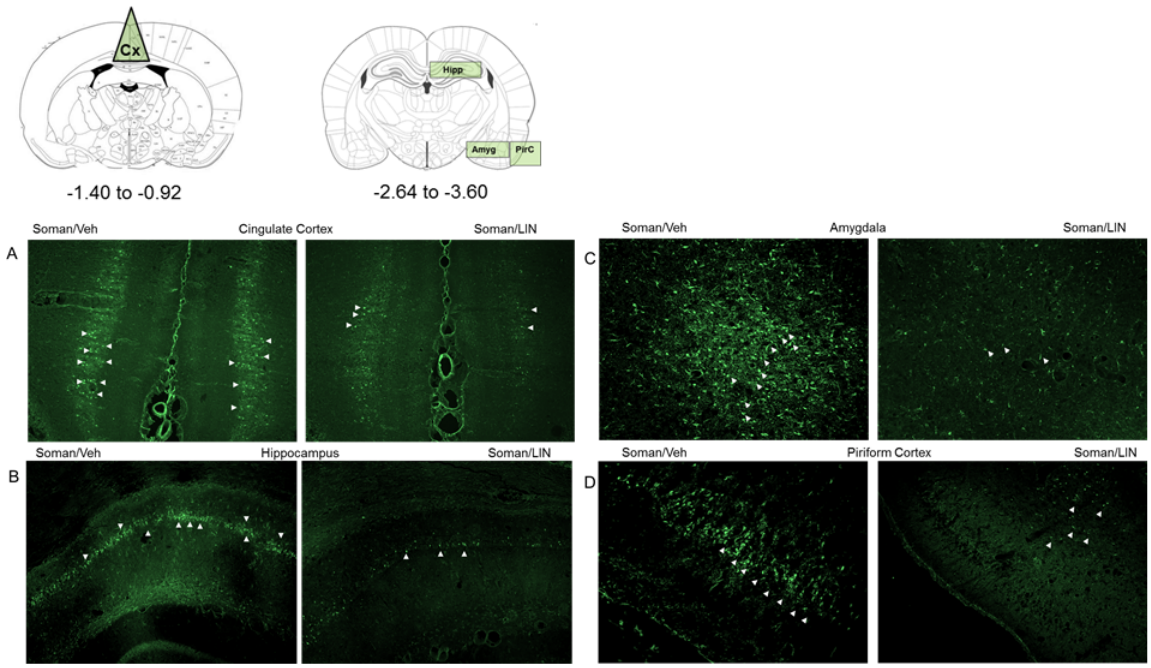
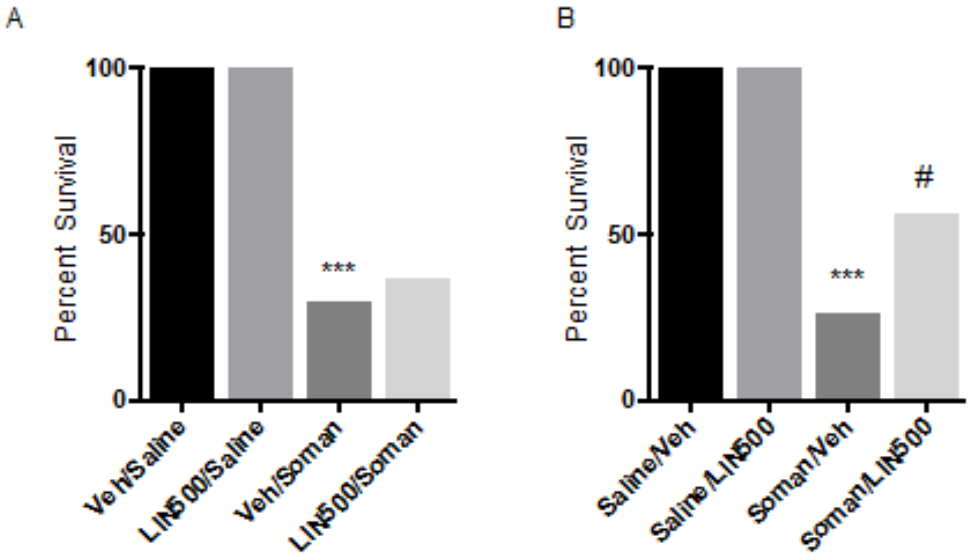


Figure 9. Effect of alpha-linolenic acid on animal survival after soman exposure.



Chapter 4: Alpha-linolenic acid-induced increase of neurogenesis is a key factor in the improvement in the passive avoidance task after soman exposure

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Keywords: alpha-linolenic acid, soman, brain-derived neurotrophic factor (BDNF), rapamycin, passive avoidance, neurogenesis, mTORC1, rat

running title: Rapamycin blocks alpha-linolenic acid-induced restorative effects in brain

ABSTRACT

Exposure to organophosphorous (OP) nerve agents such as soman inhibits the critical enzyme acetylcholinesterase (AChE) leading to excessive acetylcholine accumulation in synapses, resulting in cholinergic crisis, *status epilepticus* and brain damage in survivors. The hippocampus is profoundly damaged after soman exposure leading to long-term memory deficits. We have previously shown that treatment with three sequential doses of alpha-linolenic acid, an essential omega-3 polyunsaturated fatty acid, increases brain plasticity in naïve animals. However, the effects of this dosing schedule administered after a brain insult and the underlying molecular mechanisms in the hippocampus are unknown. We now show that injection of three sequential doses of alpha-linolenic acid after soman exposure increases the endogenous expression of mature BDNF, activates Akt and the mammalian target of rapamycin complex 1 (mTORC1), increases neurogenesis in the subgranular zone of the dentate gyrus, increases retention latency in the passive avoidance task and increases animal survival. In sharp contrast, while soman exposure also increases mature BDNF, this increase did not activate downstream signaling pathways or neurogenesis. Administration of the inhibitor of mTORC1, rapamycin, blocked the alpha-linolenic acid-induced neurogenesis and the enhanced retention latency but did not affect animal survival. Our results suggest that alpha-linolenic acid induces a long-lasting neurorestorative effect that involves activation of mTORC1 possibly via a BDNF-TrkB-mediated mechanism.

INTRODUCTION

Soman is a chemical warfare nerve agent that induces *status epilepticus* leading to brain damage in well-established brain regions including the cerebral cortex, hippocampus, amygdala and piriform cortex [16, 168]. The underlying mechanism of OP-induced toxicity is the irreversible inhibition of acetylcholinesterase (AChE), the enzyme that hydrolyzes the neurotransmitter acetylcholine (ACh), which in turn terminates cholinergic transmission. Accumulation of synaptic acetylcholine results in generalized tonic-clonic seizures and contributes to the release of glutamate and the overstimulation of glutamate receptors, predominately the N-methyl-D-aspartate (NMDA) receptor, leading to neuronal cell death [16, 17, 29, 165-167]. The neuropathology induced by OP nerve agents leads to severe cognitive deficits previously reported in rodents exposed to soman [19, 47, 49, 51, 52]. Some data indicate that the neurodegenerative process in the piriform cortex, amygdala and hippocampus underlie the pathophysiology of the cognitive deficits [19, 217]. However, increasing evidence suggests that neurogenesis may also be a contributory factor to nerve agent-induced long-term cognitive and behavioral disorders as impaired adult neurogenesis in rodents has been shown to be associated with defective spatial and contextual memory [35, 86, 229-231].

In addition to cognitive impairment, nerve agent exposure in humans has been reported to result in structural changes in the brain and long-term behavior consequences such as depression and post-traumatic stress disorder [PTSD] [24, 54, 56, 57, 173-175].Commensurate with the human data, administration of similar life-saving treatment in rodents has been unable to prevent nerve agent-induced neuropathology [34]. Thus,

current OP countermeasures are only modestly effective, supporting the need of new and efficacious therapies against OP-induced brain damage and long-term consequences.

Alpha-linolenic acid (LIN) is an essential omega-3 polyunsaturated fatty acid (PUFA) found in green leaves, seed oil (flaxseeds), beans and walnuts, can be purchased over-the-counter and has a wide safety margin [183]. The acute administration of a single dose of LIN, provides robust neuroprotection against neurodegeneration caused by kainic acid [109], transient global ischemia [111, 112] and organophosphates *in vivo* [36]. Although other polyunsaturated fatty acids (PUFAs) exhibited neuroprotective properties, LIN produced the most efficacious and reproducible effect [109].

Subchronic treatment with three sequential doses of LIN has been previously demonstrated to increase the endogenous expression of mature brain-derived neurotrophic factor (BDNF) levels in neural stem cells, cultured hippocampal neurons and in cortex and hippocampus. In the cortex and hippocampus of naïve mice, LIN also increased synaptogenesis, synaptic function and it increased neurogenesis in the subgranular zone of the dentate gyrus of the hippocampus [110]. This treatment schedule also significantly reduced infarct volume in the middle cerebral artery occlusion model of stroke and was associated with a marked improvement in animal survival (29). However, the effect of this dosing schedule in brain injury and the nature of the underlying molecular mechanisms, particularly signaling pathways that are activated in the hippocampus after LIN treatment, have not been investigated.

Adult neurogenesis is the generation of new neurons from neuronal precursors, and occurs mainly in the subventricular zone (SVZ) and in the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus [232]. Most of the newly generated neurons

die within four weeks after birth [215]. The process of differentiation, maturation and survival of newly formed mature neurons is regulated by a variety of stimuli, including neurotransmitters, hormones, neurotrophic factors, pharmacological agents and environmental factors [77-80, 233]. Brain-derived neurotrophic factor (BDNF) is a key neurotrophin in neuronal survival [212, 234-237] and has been demonstrated to increase neurogenesis [238]. Increased BDNF protein levels in the hippocampus under conditions of diet restriction have been shown to significantly improve survival of newly generated neurons, and neurogenesis is impaired in heterozygous BDNF knockout mice [214]. BDNF binds with high specificity to the tropomyosin-related kinase receptor type B (TrkB) [239]. Studies have demonstrated that the morphological maturation and survival of the newborn neurons are impaired in mice with a conditional deletion in TrkB in hippocampal neural progenitors and in immature neurons [125, 127], indicating that a BDNF-TrkB-mediated mechanism increases neurogenesis, maturation and the survival of newly formed neurons in the hippocampus.

In adult neurogenesis, the newly generated neurons integrate multiple signals, form new synapses in the hippocampus [76, 100], and are thought to have an important role in learning and memory and in adaptive responses in neurodegenerative disorders [240]. In agreement with these findings, compounds that positively influence neurogenesis are associated with enhanced performance in hippocampal-dependent learning tasks [93, 94, 159].

Despite recent intense research involving neurogenesis and behavior [95], signaling mechanisms that modulate adult neurogenesis are not well defined. One possible mechanism that may be involved in this process is the activation of the

mammalian target of rapamycin (mTOR) signaling pathway. This pathway is known to mediate several cellular processes in brain, and a mTOR-mediated mechanism has been suggested to play a major role in some behavior and cognitive effects in rodents, such as antidepressant-like activity [133, 136] and memory formation [241, 242]. Upstream activation of mTOR through Akt is regulated by neurotrophins such as BDNF via activation of its cognate receptor TrkB [243], which in turn regulates protein synthesis, mitochondrial function and autophagy [244].

The mammalian target of rapamycin is a large serine-threonine kinase that exists in two distinct heteromeric protein complexes referred to as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). The mTORC1 is sensitive to the selective inhibitor rapamycin and is activated by phosphorylation of serine^{2448} in the phosphoinositide 3-kinase (PI3K)-Akt pathway in response to growth factor stimulation [141]. Additional signaling cascades also activate mTOR [245, 246]. In the past decade, novel roles of mTOR function have emerged relating to neuronal differentiation and development [149, 247, 248]. Prolonged inhibition of the mTOR pathway by rapamycin has been shown to impair neurogenesis during development in mice [249].

The purpose of this study was to determine the effect of three sequential doses of LIN on neurogenesis in animals after soman exposure and to elucidate possible mechanisms in the hippocampus in parallel with a learning and memory task. We hypothesize that the LIN-induced increase in mature BDNF levels, through activation of TrkB, increases the activation of Akt and mTORC1 leading to an increase in neurogenesis and memory performance after soman exposure. A passive avoidance task

was used to investigate cognitive alterations related to the hippocampus following soman exposure in the presence or absence of LIN and rapamycin.

MATERIALS AND METHODS

Animals

Experiments were performed on male Sprague-Dawley rats weighing 275–350 g (Taconic Farms, Germantown, NY). The animals were maintained on a 12 h light/dark cycle and given food and water *ad libitum*. The animals were acclimatized to the animal facility for one week prior to drug treatment or surgery. The experimental protocol was approved by the Animal Care and Use Committees at the Uniformed Services University of the Health Sciences, and United States Army Medical Research Institute of Chemical Defense, and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act of 1966 (P.L. 89-544), as amended. All efforts were made to minimize the number of animals used and their suffering.

Placement of external jugular vein catheter (JVC):

All surgeries were performed under aseptic conditions. Anesthetized rats (isoflurane 2.5 – 3%, with oxygen) were placed in the dorsal recumbent position. Briefly, a 1.5–2 cm long incision was made in the ventrolateral aspect of the neck, parallel and approximately 0.5 cm lateral to midline. The external jugular vein was dissected free of surrounding tissue and stabilized with a proximally placed tie of 4-0 suture. A sterile catheter introducer was used to guide the placement of the jugular catheter. A 4-0 ligature was placed at the distal end of the catheter to secure it in place. Sterile catheters were tested

for patency by flushing with sterile saline. The catheter was tunneled subcutaneously to exit via an approximately 2.5 cm incision in the interscapular region. Wound clips and/or suture material were used to close the interscapular area. All surgeries and injections were standardized to avoid any additional variables. Sterile heparin–saline was injected using a standardized schedule to prevent clot formation within the catheter [36, 185].

Drug treatments

Alpha-Linolenic acid (LIN): LIN (Nu-Chek Prep Inc., Elysian, MN) was freshly prepared on the day of experimentation. Alpha-Linolenic acid was dissolved in ethanol at a molar concentration and then diluted in NaCl 0.9% solution to reach a final concentration of 500 μM. The pH of the solution was adjusted to 7.0 for bolus intravenous injection. Vehicle (0.05% ethanol) was prepared in an identical fashion and served as the appropriate control for LIN-treated animals [36]. The dose of LIN used in this study was 500 nmol/kg (LIN500) and was administered intravenously at 30 min, 3 days and 7 days after soman.

Soman: Male Sprague-Dawley rats receiving nerve agent were injected with the oxime HI-6 (125 mg/kg, intraperitoneally [i.p.]), an acetylcholinesterase reactivator that restores activity in molecules that have not undergone aging [5, 186], followed by soman (180 μg/kg, subcutaneously [s.c.], 1.6xLD_{50}) 30 min later. This dose of soman was chosen because it reproducibly elicits seizures in 100% of the animals tested [36]. Seizures occur within 4–8 min after soman injection. Rats were monitored for behavioral signs of seizures and peripheral effects. Atropine methyl nitrate, an inhibitor of peripheral

muscarinic acetylcholine receptors (2 mg/kg, intramuscularly [i.m.]) ,was administered 1 min after soman. Rats were allowed to seize for 40 min and then treated with anticonvulsant, diazepam (10 mg/kg, i.m.) to stop/attenuate the *status epilepticus*. The minimal duration of seizure activity necessary for irreversible damage is about 20 min, the damage process accelerates greatly after this minimal time has elapsed [187]. Atropine and HI-6 do not readily cross the blood-brain barrier, but these agents effectively block OP effects in the periphery [188]. The three drugs, HI-6, atropine methyl nitrate and diazepam, are employed to increase animal survival. After soman exposure, one group of rats was euthanized on day 10. Another group were allowed to recover for two weeks, subjected to behavioral tests and euthanized on day 31 after soman exposure.

Rapamycin: Rapamycin (LC Labs, Woburn, MA, USA) was initially dissolved in 100% ethanol to prepare a stock solution of 20 mg/ml and stored at −20°C., Rapamycin was diluted to a final concentration of 4% ethanol and 1 mg/ml rapamycin in a vehicle solution containing 5% Tween 80 (sigma), 5% PEG 400 (low-molecular-weight grade of polyethylene glycol) (Sigma) immediately prior to use as previously described [153, 250]. Both rapamycin and vehicle-treated rats received the same concentration of ethanol (4%). Rats were injected with 4.5 mg/kg (i.p.) of rapamycin (+Rap) or vehicle (+veh) for seven days beginning 30 min before soman exposure. This dose of rapamycin has previously shown to dramatically suppress mTOR activity in the cortex and hippocampus [250].

BrdU administration: BrdU is a thymidine analog, and it can be incorporated into DNA during the S-phase of the cell cycle as a substitute for thymidine. To maintain BrdU availability during a full cell cycle, rats were injected with two sequential doses of BrdU (300 mg/kg each injection [i.p.]), as previously described [110]. The BrdU was prepared in a solution of phosphate-buffered saline (PBS; pH 7.2 with 0.1% NaOH; Sigma) at a dilution of 20 mg/ml. The high dosage of BrdU was demonstrated to maximize BrdU incorporation in rats allowing specific and quantitative assessment [72]. In studies of adult neurogenesis BrdU-labeled cells are reported to undergo normal division, differentiation, migration, and integration [251].

Western Blotting

Rats were anesthetized with sodium pentobarbital (Fatal-Plus®) and decapitated. Hippocampi were dissected and tissues were sonicated in lysis buffer (1% NP-40, 20 mM Tris, pH 8.0, 137 nM NaCl, 10% glycerol, Tyr &Ser/Thr Phosphatase Inhibitor Cocktails; Upstate, Temecula, CA, USA). Protein concentrations were determined using the Bradford Comassie Blue protein assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as standard. Aliquots (75 μg) were boiled for 5 min in the presence of loading buffer (NuPAGE LDS Sample Buffer, Invitrogen, Carlsbad, CA), then placed on ice for 1 min. Each brain region was loaded in triplicate and proteins were separated on a 12% SDS-PAGE under reducing conditions using the Bio-Rad Mini-Protean 3 cell system. Proteins were transferred to nitrocellulose membranes (0.45 μm; Invitrogen). After blocking with 5% BSA in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) at room temperature for 1 h, blots were incubated overnight at 4°C with specific primary

antibodies. The primary antibodies used to examine the changes in protein expression included the rabbit polyclonal anti-BDNF (1:500 SCBT, Santa Cruz, CA), rabbit polyclonal anti-Akt, phospho-AKT Ser^{473}, mTOR, phospho-mTOR Ser^{2448} and β-actin (1:1000 Cell signaling, Danvers, MA). After washing in TBS-T, membranes were incubated with peroxidase-conjugated goat anti-rabbit antibody (1:3000; Cell Signaling Technology, Danvers, MA) for 2 h, at room temperature. After washing in TBS-T, blots were developed using enhanced chemiluminescence detection according to the manufacturer's recommendation (Pierce, Rockford, IL) and exposed to BioMax MR Film (Kodak Biomax, Rochester, NY, USA) under non-saturating conditions. Absorbance values of bands for proteins were analyzed by densitometry using Image J software (NIH, Bethesda, MD), and normalized relative to the total protein levels (ratio of phospho-/total) or β-actin absorbance value.

Immunohistochemical analyses

Immunohistochemistry was performed on 40 μm coronal brain section prepared on a cryostat (Leica Microsystems, Bannockburn, IL) and stored in cryoprotectant solution. All animals euthanized on day 10 or 31 after soman or saline injection were deeply anesthetized with pentobarbital (50 mg/kg, i.p.) and transcardially perfused with phosphate-buffered saline (PBS, 100 ml) followed by 4% paraformaldehyde (250 ml). The brains were removed and post-fixed overnight at 4°C, transferred to a solution of 30% sucrose in PBS for 72 h and frozen on dry ice prior to storage at −80°C until sectioning.

To double label BDNF (1:500, anti-BDNF (sheep polyclonal) Ab#75040, Abcam, Cambridge, MA) and NeuN (1:3000, anti-NeuN (rabbit polyclonal) Millipore ABN78, Billerica, MA), sections in a 1-in-6 series were collected from the cryoprotectant solution, washed three times for 5 min each in 0.1 M PBS, and then incubated in a blocking solution containing 10% normal donkey serum (Millipore Bioscience Research Reagents, Temecula, CA) and 0.5% Triton X-100 in PBS for 1 h at room temperature. The sections were then incubated with primary antibody diluted in a solution containing 5% normal donkey serum, 0.3% Triton X-100, and 1% bovine serum albumin overnight at 4°C. After rinsing three times for 10 min each in 0.1% Triton X-100 in PBS, the sections were incubated 1h with the secondary antibody diluted 1:2000, donkey anti-sheep IgG labeled with Alexa Fluor 488 and donkey anti-rabbit IgG Alexa Fluor 594 (Life Technologies, Carlsbad, CA). After a final rinse in PBS for 10 min, sections were mounted on slides (Superfrost Plus; Daigger, Vernon Hills, IL), air-dried, and coverslipped with ProLong Gold antifade reagent (Invitrogen, Carlsbad, CA).

BrdU immunostaining was followed according the manufacturer instructions. Briefly, sections in a 1-in-6 series were collected from the cryoprotectant and washed three times for five minutes each in 0.01M PBS (pH 7.4) with 0.1% Triton X 100. Next, sections were incubated in HCL (1N) for 10 min on ice to denature DNA, followed by HCl (2N) for 10 min at room temperature before placing them in an incubator for 20 min at 37^{o}C. The sections were incubated with 0.1M Borate buffer (pH 8.5) for 12 min and rinsed three times for five minutes each with 0.01M PBS (pH 7.4) with 0.1% TritonX100 at room temperature. The sections were then incubated with 0.01M PPBS (pH 7.4) + 0.1%

Triton X 100 + Glycine (1M) + 5% normal donkey serum for 1h prior to incubating overnight at room temperature with anti-BrdU (1:500, Sheep polyclonal, Abcam ab#1893, Cambridge, MA) and anti-Doublecortin (1:1000, Rabbit polyclonal, Abcam ab#18723, Cambridge, MA) or anti-NeuN (1:1000, Rabbit polyclonal, Millipore ABN78, Billerica, MA). The next day, the same sections were washed in 0.01M PBS (pH 7.4) with 0.1% Triton X 100 three times for five min each and incubated 2h with secondary antibody diluted 1:1000: donkey anti-sheep IgG labeled with Alexa Fluor 594 and donkey anti-rabbit IgG Alexa Fluor 488 (Life Technologies, Carlsbad, CA).

Cell Counting

Quantification of BrdU-, BrdU/DCX- and BrdU/NeuN-positive cells was performed by manually counting every sixth section throughout the hippocampus. The number of positive cells was counted in the granular and subgranular layer of the DG of hippocampus in four to six 40-um coronal sections per animal (n=4-5 per group). BrdU-positive cells were counted using an Axiovert 200 microscope with a 40× objective (Carl Zeiss International, Thornwood, NJ) and expressed as the average number of BrdU-positive cells per section [252, 253]. Control sections for the immunostaining were included and omitted the incubation of the primary antibody. Small BrdU-labeled nuclei (presumed to be glial precursors) at the hilar border and linear (endothelial-like) immunostained forms were excluded from the analysis [254]. For double labeling, the percentage of BrdU-labeled cells that expressed DCX or NeuN was determined by counts of labeled cells throughout the DG with a 60× oil immersion objective on a confocal microscope (Axio Scope 1; Carl Zeiss, Stuttgart, Germany). Only DCX-positive

immature neurons that had a minimal dendritic tree overlap with adjacent cells were included to avoid ambiguity [255].

Locomotor function and behavioral assessment

Open field test: Each rat was individually placed in a Plexiglas cage (measuring 40 x 40 x 30 cm; Accuscan Instruments Incorporated, Columbus, OH) with infrared photo cell system (Accuscan Superflex Sensor Version 2.2). When testing, the room was illuminated with two infrared lights containing 830 nm filters that is not visible to rats. The open field test was performed on day 16 and 17 after soman exposure. Rats were first acclimated for 1h (training day) and open field activity was measured on the next day (test day) for 1h. The total distance, measured in centimeters, was recorded for all groups of animals and the results for each group of animals are plotted as total distance in cm ± SEM.

Passive avoidance task: All animals were tested in a shuttlebox step-through passive avoidance task. The training day for the passive avoidance task was performed on day 16 and the test was performed on day 17 and 22 after soman exposure. The Gemini model (San Diego Instruments, San Diego, CA) consists of two (light and dark) chambers (21 cm x 25 cm x 17 cm) separated by a guillotine door. A 50 watt light bulb is present in the light chamber. On the first day (training day) a shock (0.8 mA/ one second) is delivered after the rat crosses the door and enters the dark chamber. The testing period is performed 24 hours and 5 days after the first day and the retention to cross the door (retention latency) is recorded by the computer software ("PA", San Diego Instruments,

San Diego, CA). The procedure was identical to the first day, except that the rats were not shocked upon crossing into the darkened chamber. A maximum retention latency of 300 seconds was given to rats that did not enter the dark compartment before that time. After each trial, the animals were returned to their respective home cages and the apparatus was cleaned. The retention latency of a single trial during each testing period was recorded for all animals in each group and the results were analyzed and plotted as average retention latency in seconds ± SEM.

Statistical analyses

All values were expressed as means ± SEM except where specified. Sample sizes (*n*) refer to the number of rats included in each analysis. The statistical evaluation of the results was carried out using two-way repeated measures analysis of variance (ANOVA) for changes in body weight, one-way analysis of variance (ANOVA) for neurogenesis and behavioral tests and the Fisher exact test for overall animal survival. Following significant ANOVA, *post hoc* comparisons were performed using the Bonferroni and Tukey test for a limited number of pre-planned comparisons. The level of significance for all tests is $p < 0.05$. All tests were performed using the Graphpad prism 6.0 software.

RESULTS

The experimental protocol of this study is summarized in Figure 1. Young adult Sprague-Dawley male rats received a single injection of soman (180 μg/kg; 1.6 x LD_{50}) diluted in saline with lifesaving treatment consisting of atropine, HI-6 and diazepam to increase animal survival and standardization of seizure duration. Control rats received one injection of saline, the substitute for soman, with lifesaving treatment. Alpha-

linolenic acid (500nmol/kg; LIN500) or its respective vehicle was administered 30 min, 3 days and 7 days after soman or saline injection. Rapamycin (+Rap, 4.5 mg/kg) or its respective vehicle (+veh) was administered daily for seven days beginning 30 min before soman or saline injection. Rapamycin treatment did not affect the seizure latency or duration of soman-induced seizures (Table 1).

LIN administration increases mature BDNF and activates mTOR in the hippocampus.

We first determined the endogenous expression levels of mature BDNF, phospho-Akt levels and phospho-mTORC1; the activated forms of Akt and mTORC1 respectively, in the hippocampus ten days after exposure to soman. Administration of three sequential doses of LIN500 (saline/LIN500) intravenously increased mature BDNF (mBDNF) levels about 2-fold in the hippocampus (Figure 2A-B). Exposure to soman also increased mature BDNF levels to a similar extent (Figure 2A-B, $p<0.05$) compared to saline/vehicle groups of animals. Moreover, the increase in mBDNF is significantly higher in the soman/LIN500 group ($p<0.01$) compared with the soman/vehicle or the saline/LIN500 groups of animals (Figure 2B). Phospho-Akt (activated) expression is significantly increased after LIN500 treatment in the saline/LIN500 and soman/LIN500 groups of animals compared to the saline/vehicle group of animals; soman did not increase Akt in the absence of LIN (Figure 2C). Phospho-mTOR (activated) expression significantly increased ($p<0.05$) only after LIN500 administration in the saline/LIN500 and soman/LIN500 groups compared to saline/vehicle. As observed in the activated Akt results, soman/vehicle did not increase activated mTOR. The increase in activated mTOR

was significantly higher in the soman/LIN500 group of animals compared with the saline/LIN500 groups of animals. No changes were found in the total Akt or mTOR levels.

It is known that BDNF is widely expressed in the hippocampus of adult rats [256]. Because the Akt and mTOR were activated in the hippocampus in groups administered the three doses of LIN500 but not in soman-treated groups of animals, we hypothesized that perhaps BDNF was expressed in a different subset of cells in the hippocampus of the soman/vehicle group of animals. Therefore, we undertook an immunohistochemical study to determine the cellular localization of the increased BDNF protein expression levels in the hippocampus observed in the western blots from soman/veh and soman/LIN500-treated animals. Representative confocal photomicrographs were acquired from the granular and subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus; a brain region that is known to undergo neurogenesis. In soman exposed rats, the increase in BDNF-labeled cells was mostly localized to non-neuronal cells of the DG as immunoreactive BDNF cells do not co-localize with the mature neuronal marker NeuN in the DG (Figure 2E, upper panels). In sharp contrast, the increase in NeuN co-localized with BDNF-labeled cells in the DG from animals exposed to soman followed by administration of three doses of LIN500 (Figure 2E, lower panels).

BrdU incorporation, doublecortin and NeuN expression in the hippocampus after soman exposure.

Evidence has suggested that neurogenesis appears to be critically implicated in hippocampal learning and memory [215]. We have previously demonstrated that administration of three doses of LIN increases neurogenesis in the DG of the hippocampus of naïve animals [110], however the molecular adaptive responses that may be activated after a brain insult are unknown. To address these questions, we determined neurogenesis and the effect of the mTORC1 inhibitor rapamycin in the hippocampus after administration of three doses of LIN500 in soman- exposed rats.

Quantitative evaluation of neurogenesis was examined using doublecortin (DCX), a marker of immature neurons, that co-localizes with BrdU-positive cells in the granular and SGZ of the DG of the hippocampus ten days after soman exposure. A significant increase in the percent of BrdU/DCX positive cells was only observed in the saline/LIN500 +veh [vehicle for rapamycin] ($p<0.01$) and in the soman/LIN500 +veh group of animals ($p<0.01$). In sharp contrast, rapamycin (+Rap) treatment abolished the LIN-induced neurogenesis in the saline and soman-treated animals ($p<0.05$). There is no significant alteration in neurogenesis in saline/veh +veh or soman/veh +veh or in the same groups of animals followed by rapamycin (+Rap) treatment. This result demonstrated that rapamycin does not exert an effect on basal neurogenesis in the granular and SGZ of the DG (Figure 3A). In contrast, while the average number of BrdU-positive cells was significantly higher in saline/LIN500 +veh and soman/LIN500 +veh groups ($p<0.05$) compared to saline groups of animals ten days after soman exposure, treatment with rapamycin did not significantly alter the number of BrdU-positive cells across groups of animals indicating that rapamycin does not alter cellular proliferation in this model. Since rapamycin did not significantly reduce the number of cells in the LIN-

treated groups, these results support the idea that the increased neurogenesis induced by LIN represents a small fraction of the total cellular proliferation (Figure 3B). Representative confocal photomicrographs acquired from the granular and SGZ of the DG of hippocampus ten days after soman exposure show that the increase in BrdU-labeled cells co-localize with DCX, a marker of immature neurons, after LIN500 treatment (soman/LIN500 + veh) [closed arrowheads] compared with soman/veh + veh (Figure 3C). In contrast, BrdU-labeled cells in LIN500-treated groups of animal administered rapamycin (Soman/LIN500 + Rap) do not co-localize with DCX and is similar to the BrdU-labeled cells from animals exposed to soman/Veh +veh (Figure 3C).

To investigate the possibility that the LIN500-induced increase in immature DCX-positive neurons would survive to become mature neurons, the number of mature neurons was quantified using NeuN, a marker of mature neurons. Positive cells which co-localize BrdU and NeuN were identified as mature neurons in the granular and SGZ of the DG thirty-one days after soman exposure. Consistent with the BrdU/DCX results, a significant increase in the percent of BrdU/NeuN positive neurons was observed only in groups of animals treated with LIN: saline/LIN500 +veh group ($p<0.01$) or soman/LIN500 +veh group ($p<0.05$) [Figure 3D]. Rapamycin (+Rap) treatment prevented the LIN500-induced increase in the percent of mature neurons in both groups of animals ($p<0.05$). There was no significant alteration in the number of BrdU/NeuN positive neurons in the saline/veh or soman/veh groups prior to or after administration of rapamycin (+Rap). This result demonstrates that rapamycin does not affect basal neuronal maturation (Figure 3D). The average number of BrdU-positive cells was significantly increased ($p<0.05$) in the saline/LIN500 +veh which was not affected by

rapamycin. Rapamycin treatment (+Rap) did not significantly change the average number of BrdU-positive cells in the saline groups of animals. The average number of BrdU-positive cells was significantly ($p<0.05$) increased in the groups of animals exposed to soman and rapamycin (+Rap) was without effect by rapamycin (Figure 3E). These results show that rapamycin did not affect cellular proliferation in this model and confirms findings observed in the doublecortin experiments (see Figure 3B). Representative confocal photomicrographs acquired from the granular and SGZ of the DG of hippocampus thirty-one days after soman exposure shows that the BrdU-labeled cells co-localize with NeuN [closed arrowsheads] in the LIN500-treated group of animals (Soman/LIN500 + veh). In contrast, BrdU-labeled cells in LIN500-treated groups of animal administered rapamycin (Soman/LIN500 + rap) do not colocalize with NeuN and the results are similar to the BrdU-labeled cells from animals exposed to soman/veh + veh (Figure 3F).

Locomotor behavior in the open field test

To evaluate whether the systemic administration of rapamycin (+Rap) or the vehicle (+veh) for rapamycin may affect locomotor activity in groups of rats administered soman/Veh or soman/LIN500 and saline control groups of animals, spontaneous locomotor behavior was examined on day 16 and 17 after soman exposure using the open field test. Administration of rapamycin (+Rap) or the vehicle (+veh) for rapamycin in combination with soman/veh and soman/LIN500 groups do not show any significant effect on locomotor activity as compared to saline control groups [$F(7, 32) = 0.8671$, $p= 0.5425$)] (Figure 4). These results show that the administration of rapamycin or the

vehicle for rapamycin in combination with soman and LIN500 do not affect the general health of rats.

Memory performance in the passive avoidance test

To test the effect of three sequential doses of LIN on the passive avoidance task, a widely used learning and memory paradigm, adult rats were tested on day 17 and 22 after soman exposure. Administration of rapamycin or the vehicle for rapamycin was administered systemically to further explore whether inhibition of mTORC1 affects cognitive performance after LIN treatment.

There was no significant effect in retention latency 24h after the training day across all groups of animals ($p = 0.4086$) in the passive avoidance task (Figure 5A). However, there is a significant group effect upon re-exposure to the passive avoidance task when tested day five days after training. In particular, a significant decrease in retention latency was observed in the soman/Veh +veh group ($p<0.05$) compared to the saline/Veh +veh group. The reduced retention latency induced by soman was significantly ameliorated by LIN500 treatment in the soman/LIN500 +veh group of animals ($p<0.05$) [Figure 5B]. In parallel to abolishing LIN-induced neurogenesis in the DG of the hippocampus, rapamycin treatment significantly blocked the increase in retention latency ($p<0.05$) induced by LIN500 (soman/LIN500 +Rap) in the soman-exposed rats. There was no significant alteration in memory performance in the saline-treated groups of animals that were administered rapamcin (+Rap). In the soman groups of animals, rapamycin (+Rap) did not significantly affect the retention latency in the absence of LIN500 (Figure 5B).

General health observations

Soman injection induces a statistically significant decrease in average body weight during the first three days ($p<0.05$) after soman exposure in the soman/veh +veh and soman/LIN500 +veh groups compared to saline-treated groups (Figure 6A). After this critical time of weight loss, rats recover well and all groups maintain similar weight by day 16. Rapamycin (+Rap)-treated rats tended to weigh less than saline/ Veh + veh group and average body weight was significantly different on day 16 ($p<0.05$). At the end of this experimental study, rats treated with rapamycin weighed about 25% less and were slightly smaller than rats treated with vehicle in all groups (Figure 6B).

Despite the weight loss in the rapamycin-treated groups of animals, LIN500 treatment significantly increased animal survival after soman injection ($p<0.05$). Rapamycin (+Rap) or vehicle (+veh), did not cause any alterations in survival analyzed over twenty days in any of the groups studied (Figure 6C).

DISCUSSION

This study expands on our previous findings, showing the neuroprotective effect of LIN against soman [36] and adds new information on the mechanism of neuroprotection. Our data show that administration of three sequential doses of alpha-linolenic acid increased the endogenous expression of mature BDNF and activated Akt and mTOR signaling pathways in the hippocampus analyzed ten days after soman exposure. Our proposed model is that the increase in the endogenous expression of mature BDNF results in the activation of its cognate receptor, TrkB, leading to activation of PI3K which in turn activates Akt. Akt activates mTORC1, which is required for the

LIN-induced increase in neurogenesis, the increase in the number of mature neurons and the enhancement in the performance in the passive avoidance. Rapamycin blocks the LIN-induced neurogenesis, increase in mature neurons and the improved performance in the passive avoidance task (Figure 7). The results showing that rapamycin inhibits neurogenesis in parallel with an impairment of the LIN-induced increase in retention latency and suggests a relationship whereby newly formed mature neurons are part of the adaptive response to restore hippocampal-dependent memory after soman-induced long-term cognitive deficits. These results are consistent with previous work where new neurons integrate to form functional networks [76, 89, 257]. The LIN-induced enhancement in neurogenesis in the subgranular zone of the dentate gyrus may be a crucial neurorestorative effect against soman-induced cognitive impairment.

Previous studies have demonstrated a number of diverse properties of LIN treatment, including neurotrophic factor stimulation, synaptogenesis and neurogenesis in naïve mice and as a mechanism to neuroprotection against several brain insults [109, 110, 112, 177]. In close agreement with the study of Blondeau (29), we observed that the administration of three doses of LIN significantly increased the endogenous expression of hippocampal mature BDNF levels after saline or soman exposure. Additionally, we show a possible BDNF-mediated activation of a downstream signaling pathway likely via activation of TrkB as shown by the activation of Akt which then phosphorylates mTORC1. Soman injection also triggered the endogenous expression of mature BDNF levels in the hippocampus but did not activate Akt or mTOR at the time analyzed. This differential effect may in part be due to the localization of BDNF since LIN was associated with increase neuronal expression whereas soman is associated with non-

neuronal expression. Experimental models of *status epilepticus* have been shown to stimulate the expression of BDNF mRNA and protein levels in the hippocampus and other limbic areas [258-261]. However, early studies demonstrated that the increase in BDNF expression caused by seizure agents, such as kainate, did not further activate the full-length TrkB receptor and were not neuroprotective [262, 263]. Recently, however, the role of the acute effects of BDNF expression and receptor activation in seizure models remains controversial [264-268]. Overall, our results suggest that increased mature BDNF protein levels in neurons correlate with the activation of downstream signaling possibly via activation of TrkB.

It is known that increased levels of BDNF reflect enhanced hippocampal neurogenesis [124, 126, 214]. BDNF heterozygote mice showed decreased rates of granule cell proliferation and survival, and are not influenced by enhanced environment–induced neurogenesis [269]. Conditional deletion of the TrkB gene in neural progenitor cells impairs proliferation and neurogenesis in the DG of hippocampus [127, 128] suggesting that the effect of BDNF on adult neurogenesis is likely mediated by its cognate receptor, TrkB and downstream signaling pathways. We demonstrate that LIN efficiently activated Akt, suggesting a BDNF-mediated mechanism via TrkB activation that in turn activated the PI3K pathway and Akt. Soman failed to increase activated Akt. We then tested the hypothesis that mTORC1, a downstream effector of Akt, is part of the signaling cascade involved in hippocampal neurogenesis and hippocampal-dependent forms of learning and memory

The mTORC1 signaling pathway is involved in a plethora of metabolic processes [270] and increased mTORC1 activity is implicated in a number of brain functions,

including the regulation of translation and long-lasting synaptic plasticity [147]. In the hippocampus, this process is thought to form the molecular basis of learning and memory [156]. Moreover, mTORC1 function is essential for early brain development [271], neural differentiation and proliferation during neurogenesis [148-150] and behavior [133]. Here we show that LIN treatment significantly increases mTOR activation in the saline and soman groups of animals. Soman failed to increase activated mTOR.

To investigate the involvement of mTORC1 in the regulation of the neurogenesis in parallel with the cognitive-enhancing properties of LIN treatment after soman exposure, rats received systemic administration of the mTORC1 inhibitor, rapamycin (4.5 mg/kg i.p); a dose that has been previously shown to markedly inhibit mTOR activity in the cortex and hippocampus [250] without exerting an anticonvulsant effect. Although some investigations found anti-epileptogenic effects of rapamycin treatment [151, 153], our results showed no effect of rapamycin, albeit the first dose was administered 30 min prior soman exposure, on the seizure latency or duration of soman-induced seizures (Table 1). Systemic administration of rapamycin did result in a small but significant amount of weight loss in all groups treated with rapamycin regardless of whether the animals were exposed to soman or not, which is similar to previous results [272]. Despite the weight loss, rapamycin did not affect the LIN-induced increase in animal survival and there was no effect of rapamycin on locomotor activity as determined in the open field test.

The present study shows that rapamycin inhibited LIN-induced neurogenesis in the hippocampus. Other findings have recently shown the effect of rapamycin treatment on neurogenesis. In neural stem cells (NSC) from the subventricular zone, mTOR

signaling is a key regulator of neurogenesis and mTOR inhibition by rapamycin induces a quiescence-like phenotype in neural precursors [150]. Inhibition of mTOR by rapamycin also abolishes the increase of differentiated neurons stimulated by insulin in the PI3K-Akt pathway [148]. The underlying mechanism of mTOR to increase the differentiation and survival of the newly generated neurons is not clear. Notably, recent studies demonstrate that mTORC1 modulates genes encoding the enzymes of glycolysis, pentose phosphate pathway and lipid sterol biosynthesis, generating the building blocks for anabolic cell growth [158, 273]. This process could help the development and maturation of new neurons in adult neurogenesis and cellular repair in response to adverse stimuli. Moreover, BDNF signaling through mTOR has been shown to support neuron survival by inhibiting autophagy [274]. On the other hand, mTOR dysregulation could perturb homeostasis, thus fine-tuning of mTOR has been proposed to play a pivotal role in the self-renewal of neural stem cells, proliferation and neuronal differentiation [275].

The passive avoidance test is a useful method to detect impairments in learning and memory and to test the capacity of memory retention in rats [194, 195, 276]. Cognitive alterations such as deficits in learning and memory were reported following exposure to soman in animals [49-52]. Neurogenesis has been described as a required mechanism for learning and memory performance in the passive avoidance test. Reduction of neurogenesis with a rapid X-ray ablation method impaired learning acquisition in the first 24 hours after training and also long-term memory formation up to seven days after training in a modified passive avoidance task [90]. Increased neurogenesis in the dentate gyrus of hippocampus stimulated by pharmacological compounds were associated with improved cognitive function in the passive avoidance

task in naive rodents analyzed 24 hours after the training in different foot shock paradigms [91, 92, 277] In this study, retention latency in the passive avoidance task was performed 24 hours and five days after soman exposure. No memory deficits were observed when the animals were tested 24 hours after training. However, a significant impairment in memory retention was observed in the group of animals exposed to soman five days after the training. The LIN500-induced increase in neurogenesis did not enhance performance in the absence of soman. Thus, administration of three sequential doses of LIN played a significant role in recovering from the soman-induced cognitive deficit, a process that was completely disrupted by rapamycin administration. There are two possible explanations for this finding. First, the activation of mTOR signaling by neurotrophins in the brain mediates protein synthesis-dependent synaptic plasticity [147, 156, 157] and synaptogenesis [136], which strengthens neuronal connections and helps to regulate memory storage in the brain. Three sequential doses of LIN have been shown to increase synaptogenesis and synaptic function in naïve animals [110]. Second, after analyzing our results in careful consideration with other findings, it is possible that neurogenesis may play an important role in improving learning in the passive avoidance task in the setting of hippocampal damage induced by soman. Our findings show that when mTOR is inhibited by rapamycin, the LIN-induced neurogenesis is impaired and the animals perform at a level comparable to soman treatment alone on the passive avoidance task. Because LIN treatment also influences the survival and maturation of the newborn neurons, it is possible that the late functional integration of these new cells would be required to increase hippocampal plasticity [278]. Electron microscopy studies suggest that dendritic spines of newborn neurons are attracted by pre-existing synapses

leading to remodeling the hippocampal network to form functional synapses [279]. Other investigations have demonstrated that stimulation of neurogenesis in the dentate gyrus of hippocampus by pharmacological compounds treatment was associated with improved cognitive function in the passive avoidance test after a brain insult [89, 257]. Taken together, our findings show that the LIN-induced increase in neurogenesis is a crucial event for the observed increase in memory performance after soman exposure, suggesting that positive modulation of adult neurogenesis by LIN may be involved at some level in memory performance after brain injury by soman.

Our results are distinct from a previous observation in which a significant decrease in the number of DCX-positive cells is paralleled with impaired spatial learning ability in the Morris water maze after soman exposure [35]. Differences in dose, time and duration of seizures have been shown to contribute to the degree of neuropathology [217, 218] and may account for observed differences. In this study, DCX-positive cells were analyzed at day ten after soman exposure using a soman dose of 180 μg/kg (s.c) while Joosen et al., (2009) analyzed DCX-positive neurons 28 days after soman exposure using a soman dose of 200 μg/kg (s.c). Moreover, the relationship between neurogenesis and learning have been debated in the last decade, and there is strong divergence where reduction in neurogenesis causes deficits in hippocampal-dependent learning [229, 230, 280-283]. Conversely, we found an increase in BrdU-labeling in the dentate gyrus in all soman-exposed groups of animals after thirty-one days, which were not mature neurons. One study using mice injected with soman (110 μg/kg) followed by an oxime and atropine, but not diazepam, showed increased BrdU-labeling in the dentate gyrus together with long-term glial activation up to 1 month after poisoning [86]. Other models of *status*

epilepticus have shown that the acute epileptic activity causes increased proliferation and dramatic morphologic changes in neurogenesis in the hippocampus depending on the when the neurons were born in relation to the insult [84, 284, 285].

In conclusion, our data suggest that an increase in the endogenous expression of mature BDNF in the hippocampus after LIN administration and subsequent downstream signaling via mTOR activation mediates a critical biological response of the LIN-induced neurorestorative effects (Figure 7). Notably, our results suggest that mTOR plays an important role in the LIN-induced enhancement of neurogenesis and cognitive performance in animals exposed to soman, offering a potential therapeutic target to restore neurological function under pathological conditions.

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FIGURE LEGEND

Figure 1. Schematic representation of the experimental design. A timeline is shown illustrating the sequence of events comprising the experimental plan. Young adult Sprague-Dawley male rats received a single injection of soman (180 μg/kg; 1.6 x LD50, s.c.) or saline. Alpha-linolenic acid (500 nmol/kg; LIN500, i.v.) or its respective vehicle (0.05% ethanol) was administered 30 min, 3 days and 7 days after soman or saline injection. Rapamycin (+Rap, 4.5 mg/kg, i.p.) or its respective vehicle (+veh) was administered daily for seven days beginning 30 min before soman or saline injection. BrdU (300 mg/kg; i.p.) was administered on day 9 and 10 after soman or saline exposure. Behavior experiments commenced on day 16 after soman or saline exposure. Animals were euthanized on day 10 for western blot and immunohistochemical analysis or on day 31 for immunohistochemical analysis.

Figure 2. *In vivo* effect of LIN on the expression levels of mBDNF, Akt and mTOR after soman exposure. Mature BDNF (mBDNF), activated Akt (p-Akt) and activated mTOR (p-mTOR) protein levels detected by western blot (A). Densitometric values of mBDNF (B), p-Akt (C), p-mTOR (D) protein levels relative to β-actin, total Akt and total mTOR respectively. Values are expressed as mean ± SEM. (n = 3-4/group) *$p < 0.05$ or ***$p< 0.001$ compared to saline/Veh. #$p < 0.05$ or ###$p< 0.001$ compared to soman/Veh group. Representative immunohistochemical staining of BDNF (green immunofluorescence) and NeuN (red immunofluorescence) in the granular and SGZ of the DG of the hippocampus from rats treated with LIN500 after soman exposure

(soman/LIN500) [lower panel] or vehicle after soman exposure (soman/veh) [upper panel] (E). Scale bar= 50 μm.

Figure 3. Effect of rapamycin on LIN-induced neurogenesis in soman-exposed animals. Histogram of neurogenesis analysis performed on day 10 after soman exposure in the granular and SGZ of the DG of hippocampus for all experimental groups showing the percent BrdU-positive cells that were also positive for DCX, a marker of immature neurons (A) and average number of BrdU-positive cells per section (left hippocampus) (B). Representative confocal images showing an increase in the BrdU-positive cells (red) co-localized with DCX (green) in the granular and SGZ of the DG from LIN500-treated rats after soman exposure in the presence (soman/LIN500 + rap) or absence (soman/LIN500 + veh) of rapamycin compared to Saline/Veh +veh and Soman/Veh +veh. Scale bar=20 μm (C). Histogram of neurogenesis analysis performed on day 31 after soman exposure in the granular and SGZ of the DG of the hippocampus for all experimental groups showing the percent BrdU-positive cells that were also positive for NeuN, a marker of mature neurons (D) and average number of BrdU-positive cells per section (left hippocampus) [E]. Representative confocal images showing the BrdU-positive cells (red) co-localized with NeuN (green) in the granular and SGZ of the DG from LIN500-treated rats after soman exposure in the presence (soman/LIN500 + rap) or absence (soman/LIN500 + veh) of rapamycin compared to Soman/Veh (+veh) [F]. Scale bar= 50 μm. Open and closed arrows display negative and positive co-labeled cells, respectively. Values are expressed as the mean ± S.E.M. (n = 4-5/group). *p < 0.05 or **p <0.01 compared to saline/Veh (+veh), #p <0.05 and ##p <0.01 compared to

soman/veh (+veh). +p<0.05 compared to saline/LIN500 (+veh) or soman/LIN500 (+veh) group. ns = not significant.

Figure 4. Locomotor activity in the open field test. Locomotor activity was measured by the total distance measured in centimeters (cm) traveled in the open field test of all treatment groups (n=5-7/group). Values are expressed as mean ± SEM.

Figure 5. Rapamycin prevents the LIN-induced enhancement of cognitive function after soman. Histograms from the retention latency performed 24h (A) and five days (B) after training day in the passive avoidance task. Memory impairment was induced by soman only when tested five days after training. LIN500 results in a significant improvement in retention latency which is prevented by the administration of rapamycin (+Rap) [B]. Values are expressed as mean ± SEM. (n= 5-7/group). * p <0.05 compared to respective saline groups (saline/Veh + veh vs soman/Veh + veh; saline/veh + rap vs soman/veh + rap; saline/LIN500 + rap vs soman/LIN500 + rap), #p <0.05 soman/LIN500 + veh vs soman/Veh +veh. +p<0.05 soman/LIN500 + veh vs soman/LIN500 +rap.

Figure 6. Effect of soman and rapamycin on weight and overall survival. Rats were weighed daily after soman or saline (day 0) injection throughout the duration of the experiment. Body weight is plotted over time for all treatment groups (A). A representative rat that received saline/veh +veh compared with a rat that received rapamycin (+Rap) [B]. Values are expressed as mean ± SEM (n=5-6/group). #p < 0.05 compared to saline/Veh +veh, *p < 0.05 average of rapamycin (+Rap) groups compared

to average of vehicle (+veh) group (dotted line). Overall survival for all treatment groups (C). ***$p < 0.001$ compared to saline/Veh (+veh), #$p < 0.05$ compared to soman/Veh (+veh) or soman/Veh (+rap) group. All rats that were not exposed to soman survived (n=5-8 for saline groups). The total number of animals that survived soman exposure for all treatment groups: n = 8/33 soman/Veh +veh; n = 6/10 soman/LIN500 +veh; n = 7/27 soman/Veh +Rap; n = 9/15 soman/LIN500 +Rap.

Figure 7. Proposed model for the LIN-induced neuroplastic effects administered after soman. Alpha-Linolenic acid treatment after soman exposure induces an increase in mature BDNF protein levels in the hippocampus which in turn activates its cognate receptor, TrkB resulting in (1) the activation of Akt which then activates mTORC1. Additional signaling pathways could also activate mTORC1; (2) enhanced proliferation of doublecortin-positive cells (immature neurons), and the enhanced survival of mature neurons (NeuN-positive). These actions restore memory retention in soman-treated rats. Treatment with rapamycin, an inhibitor of mTORC1, significantly blocks the LIN-induced effects on neurogenesis and memory supporting the hypothesis that LIN-induced neurogenesis is required to ameliorate the damaging effects of soman on memory in the hippocampus.

Table 1. Effect of Rapamycin in Soman-induced seizures on rats

Treatment	**Latency (s)**[a]	**% Seizures**[*]
Soman/veh/veh	161.75 ± 12.63	100
Soman/LIN500/veh	212.66 ± 80.22	100
Soman/veh/rap	165 ± 31.78	100
Soman/LIN500/rap	202.57 ± 71.65	100

Rapamycin was administered at a dose of 4.5 mg/kg daily over a seven day period.

[a]Values represent the mean ± SD, n = 3-7

[*]Seizure duration was 40 min in all groups

Figure 1. Schematic representation of the experimental design.

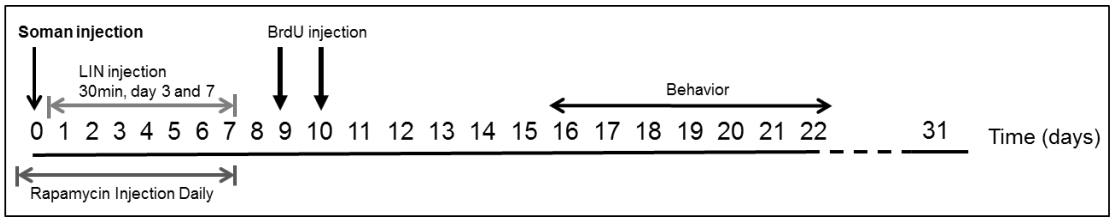


Figure 2. *In vivo* effect of LIN on the expression levels of mBDNF, Akt and mTOR after soman exposure.

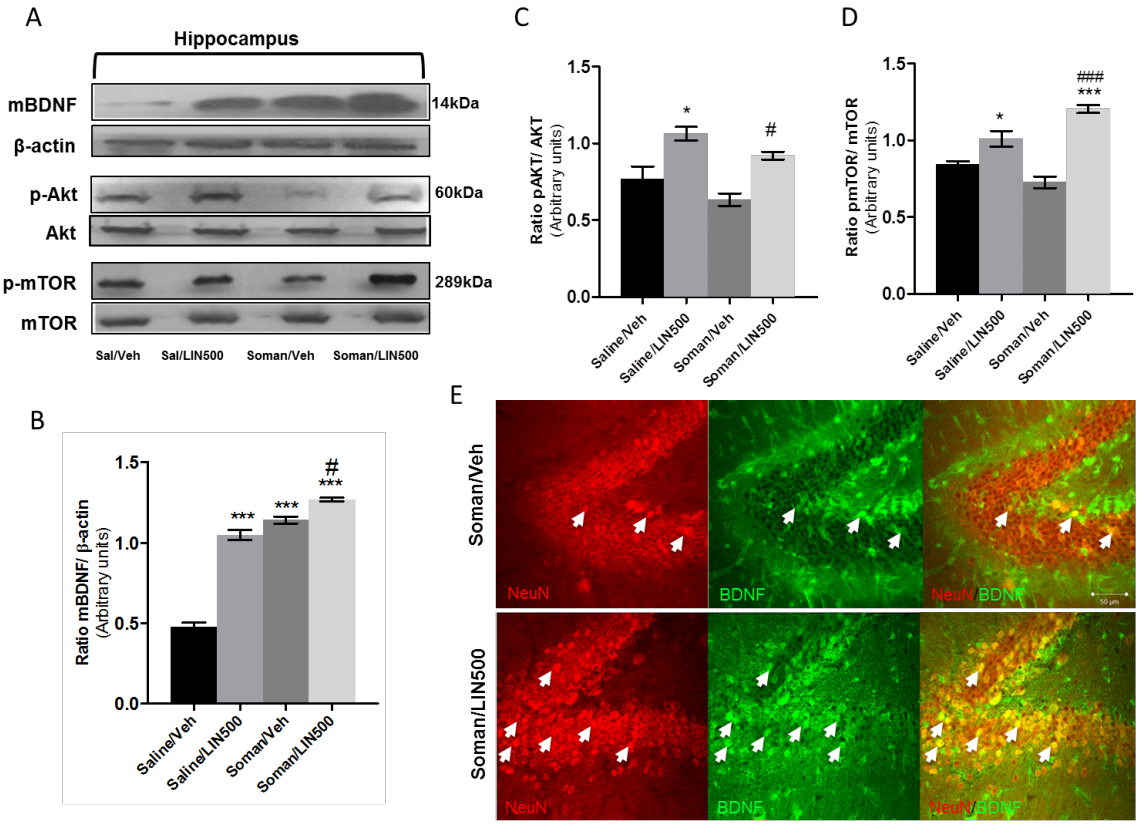
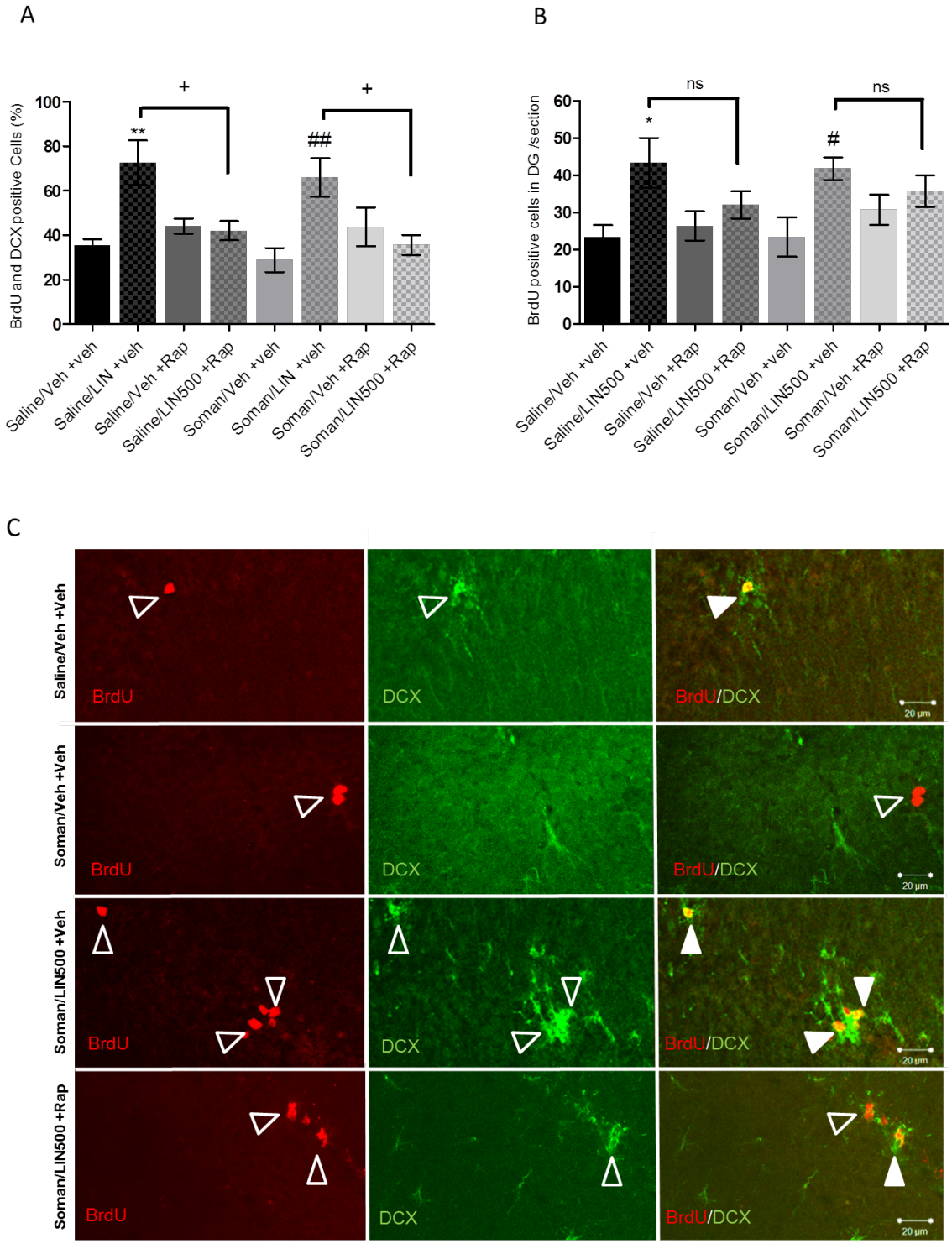
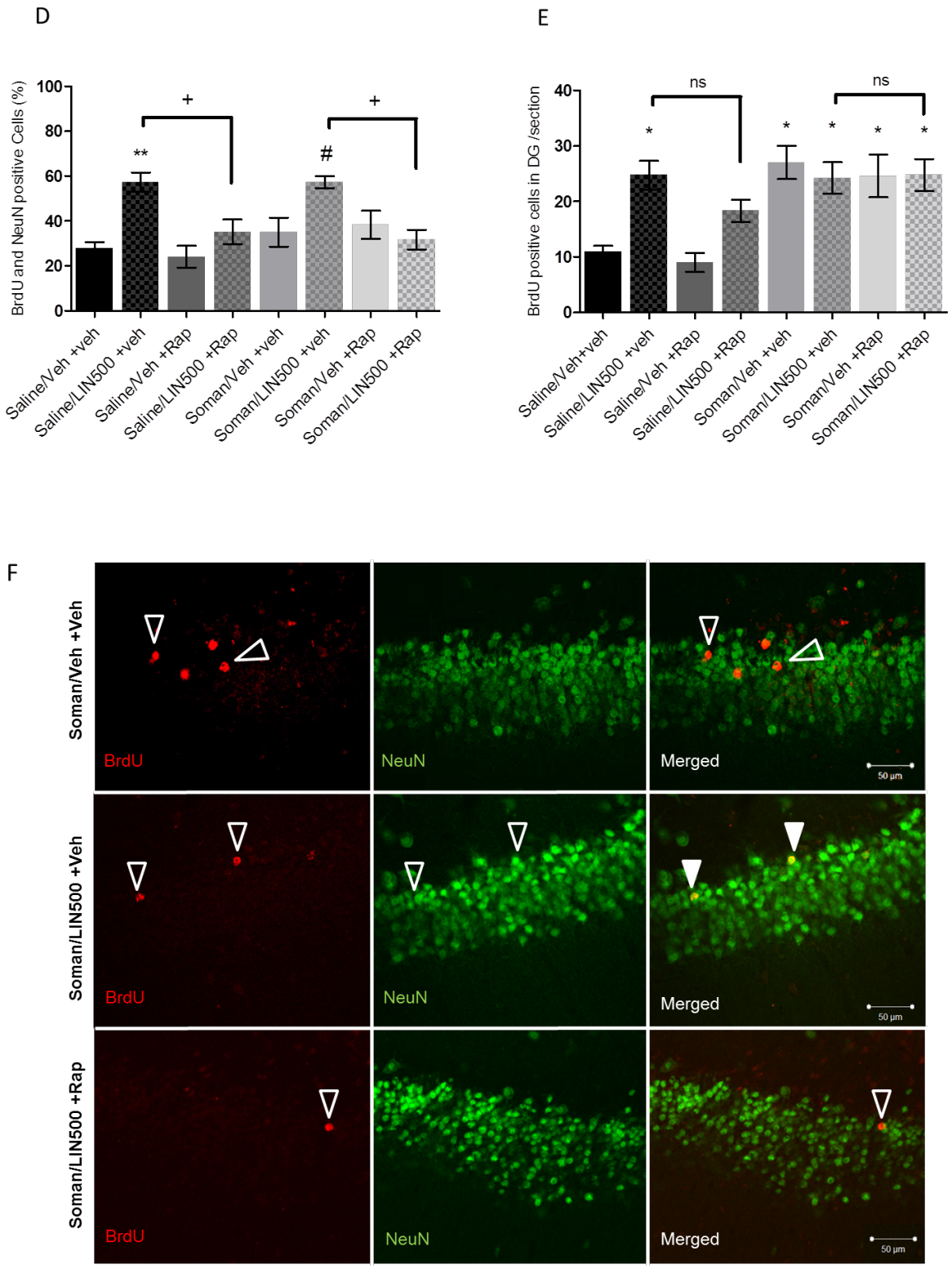


Figure 3. Effect of rapamycin on LIN-induced neurogenesis in soman-exposed animals.




D
BrdU and NeuN positive Cells (%)
100
80
60
40
20
0
+
+
**
#
Saline/Veh +veh
Saline/LIN500 +veh
Saline/Veh +Rap
Saline/LIN500 +Rap
Soman/Veh +veh
Soman/LIN500 +veh
Soman/Veh +Rap
Soman/LIN500 +Rap
E
BrdU positive cells in DG /section
40
30
20
10
0
ns
ns
*
Saline/Veh+veh
Saline/LIN500 +veh
Saline/Veh +Rap
Saline/LIN500 +Rap
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Soman/LIN500 +Rap
F
Soman/Veh +Veh
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Soman/LIN500 +Rap
BrdU
NeuN
Merged
50 µm

Figure 4. Locomotor activity in the open field test.

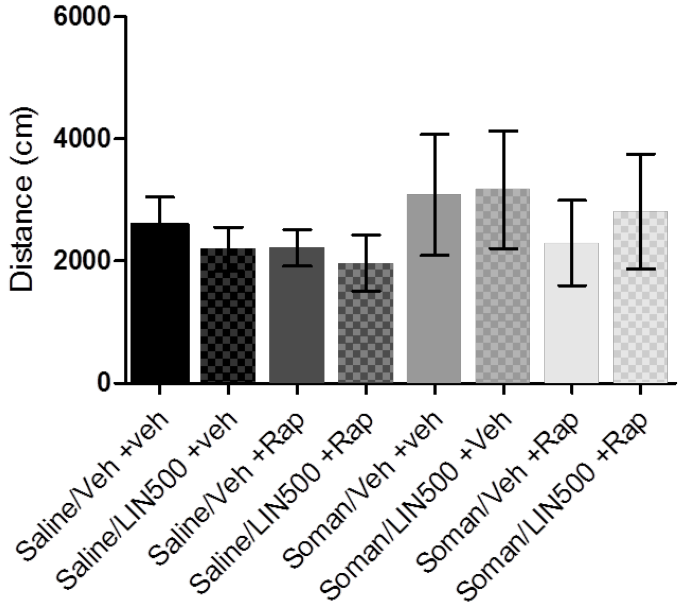


Figure 5. Rapamycin prevents the LIN-induced enhancement of cognitive function after soman.

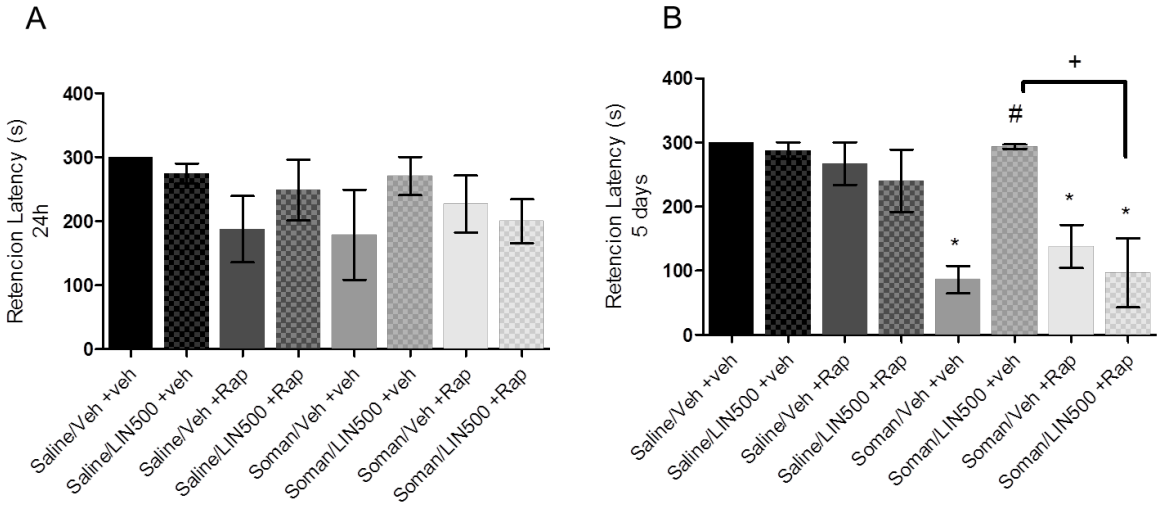


Figure 6. Effect of soman and rapamycin on weight and overall survival.

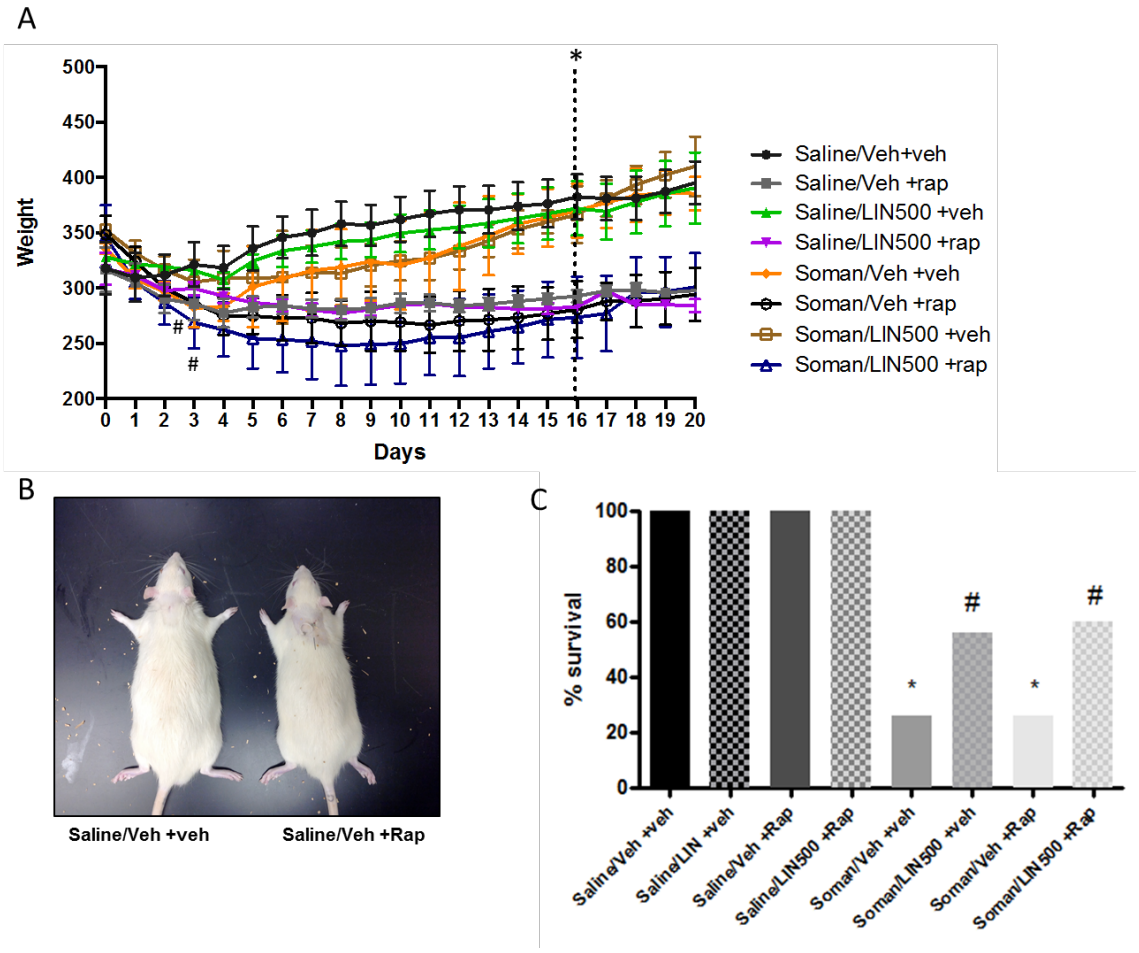
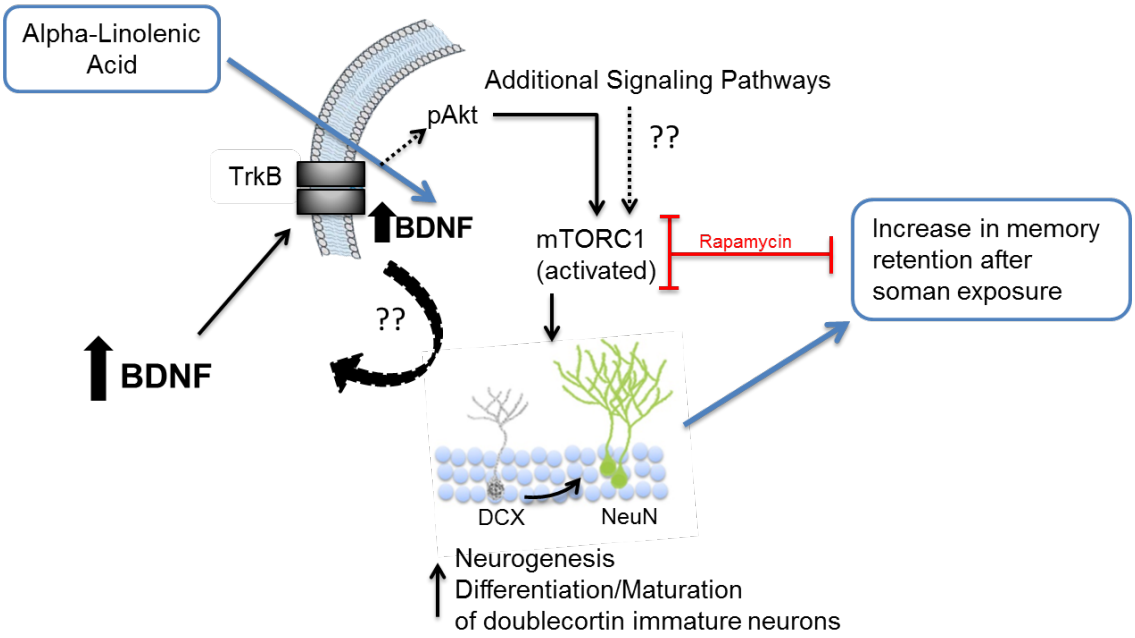


Figure 7. Proposed model for the LIN-induced neuroplastic effects administered after soman.



Chapter 5: Summary

The current study presents a novel therapeutic approach against long-term cognitive and behavioral effects of soman-induced neuropathology. Additionally, this study elucidated new molecular mechanisms involved in LIN-induced neuroprotection, and novel pathways involved in neurogenesis and memory.

First, subchronic LIN treatment has long-lasting neuroprotective effects against soman-induced neuropathology. The timing of treatment is essential for the enduring effects of LIN treatment. [chapter 3; manuscript 1]. Administration of three doses of alpha-linolenic acid injected intravenously over a seven day period *after* soman significantly improved motor performance on the rotarod, enhanced memory retention, exerted anti-depressant-like activity and increased animal survival. This dosing schedule significantly reduced soman-induced neuronal degeneration in four major vulnerable brain regions up to twenty-one days.

Follow-up studies from deployed veterans exposed to nerve agents [54, 56] and victims of the terrorism attack in Japan [55] have demonstrated neurologic and neuropsychiatric disorders involving cognitive and emotional behavior. The proposed major contributor for long-term behavioral impairments is the secondary neuronal damage after nerve agent exposure. The secondary damage is the cascade of progressive neuronal injury and neuronal cell death that is activated by the initial insult, and includes the excessive influx of calcium – which activates lipases proteases, phosphatases, and potentially other harmful cascades essential for neuronal survival, extensive cerebral edema, increased blood-brain permeability, cerebral hemorrhages, oxidative stress and increased neuroinflammation (elevated levels of cytokines and COX-2) [20].

It is not yet clear what mechanisms are activated by LIN to reduce soman-related neuronal degeneration, secondary neuronal damage, and behavior deficits. The primary effects of LIN treatment in the initial phase of soman exposure may be a reduction in NMDA receptor-mediated excitotoxicity via the activation of background rectifying potassium channels – TREK-1, TREK-2 and TRAAK [109]. LIN treatment has also been demonstrated to exhibit antioxidant and anti-inflammatory properties [183], which may contribute to its long-lasting effect to improve overall survival. More detailed characterization of the cellular mechanisms regulated by LIN treatment may have implications in the treatment of nerve agent-induced neuropathology by opening new avenues of drug discovery.

Also, a novel mechanism of LIN-induced neuroprotection was identified through activation of the Akt-mTOR pathway, which is involved in neurogenesis and improved learning and memory [chapter 4, manuscript 2]. The mTOR is ubiquitously expressed in the brain, and its activation regulates new protein synthesis leading to an increase in synaptic signaling proteins and enhancement of synaptic plasticity [286]. The knockouts of S6K1, a downstream effector of mTORC1, demonstrated abnormal fear conditioning and deficits in hippocampal learning [287]. Additionally, mTOR has recently been demonstrated to exert a critical role in early brain development [271] and in the formation of the dentate gyrus, a critical brain region involved in adult neurogenesis [249].

The discovery that neurogenesis occurs in the adult brain, raises the hypothesis that the generation of new neurons could help compensate for the loss of neurons in neurodegenerative processes from soman exposure. Although neurogenesis is a process that occurs throughout life, many of the new neurons die soon after birth. According to

Lledo et al [232], "adult neurogenesis is a wasteful process — of the newborn cells that migrate, differentiate and mature successfully, only ~50% survive for longer than a month". So far only a limited number of external stimuli have been identified, including physical activity, enriched environment, antidepressant agents and mature BDNF that are capable of improving the rate of survival of these new neurons [214, 232]. Thus, a new approach to modulate neurogenesis may provide a better understanding of the adaptive responses of the brain, particularly the hippocampus, offering a high potential therapy to relieve the cognitive and disability effects after soman exposure.

The Akt-mTOR pathway has recently been demonstrated to be activated in neuronal progenitors [288]. One of the most prominent questions is: how does mTOR contribute to the survival of the new neurons? A recent study may have begun to answer this question. Smith et al 2014 [274], using primary cultures of dissociated hippocampal neurons prepared from E18 Sprague-Dawley rats, found that BDNF signaling through Akt- mTOR, but not MAPK, is necessary to maintain neuronal survival by modulating autophagy. Autophagy is a process where amino acids, macromolecules, and damaged organelles are degraded and recycled [286]. According to Smith et al [274], addition of rapamycin to BDNF-treated neurons in culture significantly increased autophagic flux , which is the overall rate of conversion of protein to metabolites, leading neurons to die. The authors further confirm this result showing that genetic reduction of autophagy blocked the harmful effects of rapamycin. Moreover, another study demonstrated that mTORC1 modulates genes by encoding the enzymes of glycolysis, pentose phosphate pathway and lipid sterol biosynthesis, generating the building blocks for anabolic cell growth [158, 273]. Together, these processes could be mediating the development,

survival and maturation of new neurons in adult neurogenesis. The late functional integration of these new cells would be relevant to restore hippocampal plasticity and improve learning and memory after soman exposure.

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Appendix: Other therapeutic strategies against soman

We have also sought additional therapeutic strategies against soman exposure [185]. To this end, we have used (-)-phenserine, a selective competitive inhibitor of AChE and butyrylcholinestare, currently used in the treatment of Alzheimer Disease because of its dual action in the inhibition of acetylcholinesterase and reduction in the production of the Aβ precursor protein [289].

Because soman exposure results in the irreversible binding and aging of the active site of AChE, pretreatment with a reversible inhibitor such as (-)-phenserine before soman exposure, would provide a temporary shield to the active site of the AChE. Similar strategies with other reversible anticholinesterases, including huperzine [290], carbamates [291] and galantamine [292], have demonstrated successful approaches against nerve-agent exposure. However, (-)-phenserine has several additional prospective advantages: it is highly lipophilic and therefore has a very high brain to periphery ratio, low toxicity, and unlike many clinical anticholinesterases, does not induce changes in AChE expression [289, 293, 294]. Moreover, administration of (-)-phenserine has demonstrated rapid, potent and long-lasting AChE inhibition and improvement of cognitive performance in both young learning-impaired and elderly rats [289].

We aimed to evaluate the neuroprotective function of (-)-phenserine against soman-induced neuropathology. Soman-induced seizures (180 μg/kg; s.c.; 1.6xLD_{50}) resulted in significant mortality, movement dysfunction and neurodegeneration of male Sprague-Dawley rats. Treatment with (-)-phenserine (1mg/ml; i.v) prior to soman exposure, increased survival rate, improved movement and reduced neurodegeneration. Moreover, (-)-phenserine post-treatment improved neuronal survival at a lower but

significant level, whereas this dosing schedule did not impact survival rate or movement recovery, suggesting that (-)-phenserine provides neuronal protective functions independent from its AChE binding [295].

To identify molecular changes from soman-induced brain damage, quantitative PCR was used to evaluate gene expression profiles three hours after soman exposure in the piriform cortex. This brain region stands out as one of the central regions in the induction of seizures and consequent neurodegeneration in response to nerve agent [296]. The (-)-phenserine 30 minute pretreatment was analyzed as this pretreatment time afforded the greatest protection based upon animal survival and neuropathology. Selected genes of interest were restricted to expression in neurons and involved in programed cell death, cell survival, or signal transduction pathways. Administration of (-)-phenserine thirty minutes prior to soman modulates gene expression in the piriform cortex three hours after soman. We found 17 target genes, reported in neuroprotective functions [e.g., AID genes (Atf3, Btg2, Gadd45g), Nr4a1, Fos, Cited2, Dusp1, Klf4, and Nfil3], associated with neuronal apoptosis [e.g. Nos3, Crh, Crem, Homer1] and inflammatory markers [Ccl3 and TNF]. Also, Abra (also known as Stars) and Calca expression profiles were evaluated. Together these results are a preliminary analysis to elucidate molecular pathogenesis of soman-induced brain damage, providing a stage for the development of neuroprotection drugs and the understanding of (-)-phenserine's neuroprotective actions.

In conclusion, acute (-)-phenserine treatment demonstrated significant protection against soman-induced neuronal cell death, motoric movement and reduces mortality when given as a pretreatment. (-)-Phenserine also protects vulnerable neurons when given

after soman. Future research may investigate long-term effects of (-)-phenserine, based on the modulation of genes critically involved in the pathophysiological as well as protective pathways in neurons [295].

In the event lacking first-aid emergency care such as after the terrorist attack of the Tokyo subway [23, 297] and Syrian attack (http://www.theguardian.com/world/2013/sep/16/un-inspectors-syria-sarin-gas) or from accidental poisoning as occurred in India (http://edition.cnn.com/2013/07/17/world/asia/india-school-meal-poisoning/), long-term term consequences of nerve-agents exposure are often debilitating. Thus, there is a pertinent need for pharmacological approaches that allow subsequent use. In this scenario, LIN and (-)-phenserine treatment offer a safe, effective and long-term strategy against nerve-agent exposure.